PURIFICATION AND CHARACTERIZATION OF PEROXIDASES FROM BITTER GOURD (*Momordica charantia*)

SUMMARY

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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BY

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Summary

Bitter gourd (*Momordica charantia*) is an important medicinal plant, which is easily available in most parts of India. Plant peroxidases are receiving increasing attention due to their extensive bioactivation property and potential applications in clinical, biochemical, biotechnological, and related areas.

Peroxidase from bitter gourd was purified by ammonium sulphate fractionation followed by gel filtration and affinity chromatography. The enzyme was purified to 42 fold with the retention of 67% of the initial activity. The enzyme exhibited its maximum activity at pH 5.6 and 40 ^{o}C. The enzyme retained half of its activity after 1 h incubation at 60 ^{o}C. Molecular weight of the purified glycosylated bitter gourd peroxidase determined by Sephacryl S-100 and SDS-PAGE was 43 kDa. Stokes radius, diffusion coefficient and sedimentation coefficient of the purified peroxidase were 27.3 Å, 8.17 x 10^{-7} cm^2/sec and 3.74 S, respectively. K_m for *o*-dianisidine and ABTS were 1.3 and 4.9 mM, respectively. The activity of the enzyme was inhibited by sulfide, azide and L-cysteine. The sulphydryl groups of the enzyme were 16 mmoles/mole of the protein. Bitter gourd peroxidase shares its properties with the typical class III peroxidases.

Glycosylation has been shown to play an important role in protein folding, biological activity, protein stability and immunogenicity. Majority of plant peroxidases have been reported to be glycosylated. The purified bitter gourd peroxidase obtained as a single band was found to be glycosylated whereas the two other isoenzymes obtained were non-glycosylated. The carbohydrate content of purified bitter gourd peroxidase was 25% (w/w) mass of the protein. Purified bitter gourd peroxidase gave a pink colored band when stained by periodic acid schiffs reagent.

The possible role of carbohydrate moieties in the stabilization of proteins was investigated by using bitter gourd peroxidase as a model system. A comparative study of glycosylated and non-glycosylated isoenzymes of bitter gourd peroxidase was performed at various temperatures, pH, water-miscible organic solvents, detergents and chaotropic agents; like urea. The pH and

temperature-optima of both glycosylated and non-glycosylated isoforms of bitter gourd peroxidase remained unchanged. Glycosylated bitter gourd peroxidase retained more activity than non-glycosylated preparation at various temperatures, other than the temperature-optima. Glycosylated bitter gourd peroxidase was also significantly more stable than non-glycosylated preparation when incubated at different time intervals at 60 °C. The glycosylated form of bitter gourd peroxidase retained a higher enzyme activity as compared to the non-glycosylated bitter gourd peroxidase when exposed to various detergents, SDS. Tween-20 and Triton X-100. Glycosylated bitter gourd peroxidase also retained greater fraction of catalytic activity when exposed to various water-miscible organic solvents like dimethyl sulphoxide and dimethyl formamide. Thus. glycosylated bitter gourd peroxidase retained significantly more catalytic activity against the exposure caused by various physical and chemical denaturants. Non-glycosylated bitter gourd peroxidase exhibited higher intrinsic fluorescence intensity as compared to glycosylated bitter gourd peroxidase due to greater exposure of fluorophore in this form. Unfolding of both forms of bitter gourd peroxidase in the presence of high urea concentrations, studied by fluorescence measurement indicated greater perturbations in the conformation of non-glycosylated preparation. The different properties examined thus indicated that glycosylation played an important role in the stabilization of native conformation of proteins against the inactivation caused by various types of denaturants.

Horseradish peroxidase has dominated the world of peroxidases since a long time. It is widely used in diagnostic, biosensing and biotechnological applications. However, the availability and high cost of commercially available horseradish peroxidase restricts its applications. The structural and functional aspects of bitter gourd peroxidase and horseradish peroxidase have been compared for their stability against the denaturation induced by heat, pH, urea, SDS, and water-miscible organic solvents. Stability of the enzymes was monitored spectrophotometrically as well as by ellipticity changes in far and

near UV-CD region. Bitter gourd peroxidase was more thermo-stable as compared to horseradish peroxidase. Temperature activity profiles of bitter gourd peroxidase and horseradish peroxidase exhibited similar temperature-optima at 40 °C. Bitter gourd peroxidase retained a higher fraction of catalytic activity as compared to horseradish peroxidase when incubated at various temperatures (30-80 °C). The disruption of secondary and tertiary structure at various temperatures was greater for horseradish peroxidase. The secondary structure of bitter gourd peroxidase remained stable till 72 °C whereas horseradish peroxidase lost substantial secondary structure from 60-80 °C. This observation was further supported by far UV-CD spectra of bitter gourd peroxidase at 30 °C, 60 °C and 80 °C. Bitter gourd peroxidase retained remarkably greater fraction of enzyme activity as compared to horseradish peroxidase in the alkaline pH range. The difference in catalytic activity of bitter gourd peroxidase by varying the pH was related to the change in secondary structure as manifested by the change in the CD value at 222 nm. It was further complemented by the far UV-CD spectra, which showed greater retention of secondary structure at pH 6.0 and pH 10.0. Bitter gourd peroxidase showed remarkable stability in the presence of urea. There was no change in the enzymatic activity of both bitter gourd peroxidase and horseradish peroxidase till 2.0 M urea exposure. However, there was a significant decrease in enzymatic activity beginning from 4.0 M urea. Bitter gourd peroxidase retained greater fraction of catalytic activity when exposed to 4.0 M urea for varying times. The retention of catalytic activity by bitter gourd peroxidase was remarkably greater than horseradish peroxidase when exposed to higher concentration of urea (8.0 M) for 2 h. Bitter gourd peroxidase and horseradish peroxidase exhibited an enhancement in enzyme activity when exposed to SDS. However, the enhancement in enzyme activity was more for bitter gourd peroxidase as compared to horseradish peroxidase. Both peroxidases were activated by the exposure to 20-60% (v/v) of dimethyl formamide. However, there was more activation in enzyme activity of bitter gourd peroxidase as

compared to horseradish peroxidase activity. Bitter gourd peroxidase activity was markedly enhanced and it was more stable than horseradish peroxidase on exposure to 10-60% (v/v) dimethyl suphoxide. In view of its higher stability, bitter gourd peroxidase can serve as a better alternative to horseradish peroxidase in clinical, biochemical, analytical, and environmental analyses as well as in various biotechnological applications.

Purified bitter gourd peroxidase was injected into healthy male albino rabbits for the production of anti bitter gourd peroxidase polyclonal antibodies. Antibodies raised against bitter gourd peroxidase were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate fractionated antiserum was passed through DEAE-cellulose column. The fractions containing purified antiperoxidase antibodies were pooled for further use. DEAE-cellulose purified IgG loaded on SDS-PAGE clearly showed two bands corresponding to the heavy and light chains of the antibody. The position of migration of IgG subunits correspond to apparent molecular weights of 45 kDa and 20 kDa. The native PAGE showed a single band and this result supported the purity of the purified antibodies. Purified anti-bitter gourd peroxidase antibodies gave a clear precipitin line with the purified bitter gourd peroxidase when Ouchterlony double immunodiffusion was performed. A high titer was obtained through direct binding enzyme linked immunosorbent assay.

The IgG isolated were used for the construction of Sepharose 4B-anti bitter gourd peroxidase immunoaffinity support. Polyclonal antibody bound Sepharose 4B support was exploited for the immobilization of bitter gourd peroxidase directly from ammonium sulphate precipitated proteins. Immunoaffinity immobilized bitter gourd peroxidase exhibited high yield of immobilization. The immunoaffinity immobilized bitter gourd peroxidase preparation exhibited very high effectiveness factor (η) value of 0.96. Immobilized enzyme exhibited no change in temperature-optima between 30-40 °C whereas soluble bitter gourd peroxidase had temperature-optima at 40

°C. Immunoaffinity bound bitter gourd peroxidase retained greater fraction of enzyme activity on both sides of temperature-optima compared to its soluble counter part. IgG-Sepharose 4B bound bitter gourd peroxidase showed a higher stability against heat, pH, chaotropic agents (urea and guanidinium chloride), detergents (Cetyl trimethyl ammonium bromide and Surf Excel), proteolytic enzyme (trypsin) and water-miscible organic solvents; propanol, tetrahydrofuran and dioxane. The activity of immobilized bitter gourd peroxidase was significantly enhanced in the presence of cetyl trimethyl ammonium bromide and after treatment with trypsin as compared to soluble enzyme.

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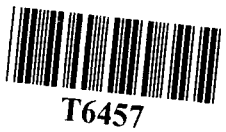

Dedicated to my Parents

To my husband – Suhail

For showering my life with happiness

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Certificate

This is to certify that the thesis entitled ***"Purification and characterization of peroxidases from bitter gourd (Momordica charantia)"*** *being submitted by Aiman Fatima to A. M. U. Aligarh for the award of the degree of Doctor of Philosophy in Biochemistry is a record of bonafide research work carried out by her. Aiman Fatima has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis.*

The results contained in this dissertation have not been submitted in part or in full to any other University or Institute for the award of any degree.

Q Husain

Prof Qayyum Husain

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'What lies behind us and what lies before us are small matter compared to what lies within us'- Ralph Waldo Emerson

Each step that I took was showered with blessings by the Almighty Allah. He has always kept me going.....

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(Aiman Fatima)

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate)
BGP	Bitter gourd peroxidase
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CNBr	Cyanogen bromide
Con A	Concanavalin A
CTAB	Cetyl trimethyl ammonium bromide
DEAE	Diethyl aminoethyl
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DTNB	5, 5' dithiobisnitrobenzoic acid
ELISA	Enzyme linked immunosorbent assay
Far UV-CD	Far ultra violet-circular dichroism
F_i	Intrinsic fluorescence
GdnCl	Guanidinium chloride
GlcNAc	N-acetylglucosamine
His	Histidine
HRP	Horseradish peroxidase
I-BGP	Immobilized bitter gourd peroxidase
kDa	Kilodalton

K_m	Michaelis-Menton constant
LiP	Lignin peroxidase
LPO	Lactoperoxidase
M	Molar
Mdeg	Millidegree
MnP	Manganese peroxidase
MPO	Myeloperoxidase
M_r	Molecular weight
Near UV-CD	Near ultra violet-circular dichroism
nm	Nanometer
NMR	Nuclear Magnetic Resonance
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid schiffs
R_z	Reinheitzahl value
SAC	Substrate access channel
S-BGP	Soluble bitter gourd peroxidase
SDS	Sodium dodecyl sulphate
TBS	Tris buffer saline
TBS-T	Tris buffer saline Tween-20
THF	Tetrahydrofuran
TOC	Total organic carbon
v/v	Volume by volume
w/v	Weight by volume

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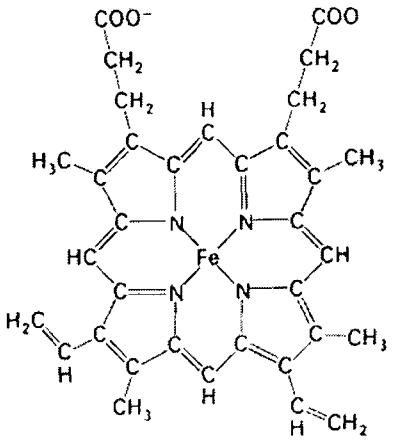
Chapter I

Review of literature

1.1 PEROXIDASES

Peroxidase (E.C. 1.11.1.7) is a heme-containing enzyme that is widely distributed in plants, microorganisms and animals (Duarte Vazquez *et al.*, 2003a). Heme is a complex between an iron ion (Fe^{+3}) and the molecule protoporphyrin IX. The iron is coordinated to four pyrrole nitrogens of the heme and nitrogen of an axial histidine.



Structural formula of heme

Peroxidases can oxidize a variety of substrates in the presence of hydrogen peroxide (Vianello *et al.*, 1997). The catalytic process of peroxidases occurs through a multi-step reaction (Banci, 1997). The steps are as follows:

1. Heme [Fe(III)] $_{PX}$ + H_2O_2 → Heme [O=Fe (IV)-$R^{+\cdot}$] $_{(Comp\ I)}$ + H_2O

   $_{PX}$ stands for peroxidase.

The active site of the peroxidase reacts with hydrogen peroxide. This results in the reduction of H_2O_2 to water and oxidation of peroxidase by two electrons. The latter state of the peroxidase is called Compound I which contains an oxyferryl [Fe (IV) =O] center and an organic cation radical that can be located either on the heme or on the protein residue, depending on the isoenzyme.

2. Heme [O=Fe (IV)-$R^{+\cdot}$] $_{(Comp\ I)}$ + S → Heme [O=Fe (IV)] $_{(Comp\ II)}$ + $S^{\cdot}$

Compound I oxidizes one substrate molecule, 'S' to give a substrate radical, 'S·' and Compound II, where the organic cation radical is reduced to its resting state.

3. Heme [O=Fe (IV)] $_{(Comp\ II)}$ + S → Heme [O=Fe (III)] $_{(PX)}$ + S·

Finally, Compound II is reduced by a second substrate molecule to the resting iron (III) state.

Peroxidases have molecular weights ranging from 35-100 kDa (Banci, 1997). The protein sequences of plant peroxidases are characterized by the presence of highly conserved amino acids such as two distal and proximal histidine residues interacting with the heme. The distal histidine is necessary for catalytic activity. These histidine residues are present in all known heme-containing peroxidases (Passardi *et al.*, 2004). Peroxidases possess a signal peptide necessary for routing to the endoplasmic reticulum, and some of them possess a C-terminal extension, which may be responsible for vacuolar targeting, as has been shown for some plant proteins (Neuhaus, 1996). The removal of C-terminal extension has been deduced from the mature cationic horseradish and barley grain peroxidases (Johansson *et al.*, 1992). In peroxidases, hydrogen-bonding networks are present on both proximal and distal side of the heme (Thanabal *et al.*, 1988).

1.1.1. Classes of peroxidases

Peroxidases have been essentially classified into three major classes depending on the organism (Welinder, 1992a). It has been proposed from an extensive comparison among the amino acid sequences, that heme peroxidases from plants, fungi and bacteria are evolutionary related and show 50% to 95% sequence homology (Welinder, 1992a; 1992b; Welinder and Gajhede, 1993).

1.1.1.1. Class I peroxidases

These are intracellular prokaryotic peroxidases such as cytochrome C peroxidase, ascorbate peroxidase, bacterial, and fungal catalase peroxidases (Hiner *et al.*, 2002). They do not contain any disulfide bridge, carbohydrate moieties or calcium

ions (Banci, 1997). Ascorbate peroxidase regulates the level of intracellular hydrogen peroxide (Mittler, 2002). From the point of view of the reaction of peroxidases with H_2O_2, the bacterial and fungal class I enzymes known as catalase-peroxidases are most intriguing (Hiner *et al.*, 2002). These enzymes possess striking heme pocket sequence homology with other class I peroxidases, cytochrome C peroxidase and ascorbate peroxidase including the proximal and distal histidines and tryptophans and distal arginine (Zamocky *et al.*, 2001). The catalase-peroxidases exhibit wide spectrum catalytic activity at a similar level to the classical monofunctional catalases (Regelsberger *et al.*, 2000).

The compound I formation in this class is extremely unstable and is rapidly converted to compound II without the addition of reducing substrate (Patterson and Poulos, 1995). It is believed that peroxidases like ascorbate peroxidase reduces its substrate which scavenges excess H_2O_2 formed in plant cells under normal and stress conditions, as do glutathione peroxidases in mammals, cytochrome C peroxidase and NAD(P)H peroxidase in bacteria. The oxidation product, monodehydroascorbic acid radical does not play any physiological role. Class I peroxidases have high specificity for ascorbate (Asada, 1992). The proximal tryptophan is hydrogen-bonded with an asparate residue, which itself hydrogen bonds with histidine proximal heme ligand, i.e. ascorbate peroxidase (Patterson and Poulos, 1995).

1.1.1.2. Class II peroxidases

These are extracellular fungal peroxidases. They are characterized by the presence of disulfide bridges, calcium ions and glycosylation on the protein surface. Tyrosine residues are absent in this class except one residue in one isoenzyme of lithium peroxidase (Banci, 1997).

1.1.1.3. Class III peroxidases

These are secretary plant peroxidases, which are present in extracellular space or vacuole (Tognolli *et al.*, 2002). They are also present in cell wall (Welinder, 1992a). The common peroxidases of this class include horseradish peroxidase (HRP), turnip peroxidase (TP) and soybean peroxidase (SBP). These peroxidases have four

conserved disulfide bridges and two structural Ca^{2+} ions (Welinder, 1979; Schuller *et al.*, 1996). Majority of such peroxidases are also glycosylated in nature (Lagrimini *et al.*, 1987: Johansson *et al.*, 1992). The class III peroxidases exhibit very low ascorbate-oxidation activity (Chen and Asada, 1989). In this class the his-asp-trp H-bonded triad is not conserved because these enzymes contain a non-polar phenylalanine at the position homologous to the proximal trp of ascorbate peroxidase of class I peroxidases (Welinder, 1979; Schuller *et al.*, 1996). Class III peroxidases have molecular masses in a range of 28-60 kDa (Hiraga *et al.*, 2001). Guaiacol is commonly used as reducing substrate of class III peroxidases and hence they are also called as guaiacol peroxidases (Kvaratskhelia *et al.*, 1997). The class III peroxidases have an ER signal peptide (Welinder, 1992b). This class is characterized by very stable compound I formation. These enzymes oxidize a wide range of substrates and the oxidation products are involved in important biosynthetic processes (Gazaryan and Lagrimini, 1996). These plant peroxidases are believed to function in diverse physiological processes including disease resistance and wound response (Sasaki *et al.*, 2004).

The peroxidases of this class are involved in cell wall construction and were found in most of the major divisions of land plants, except green algae (Passardi *et al.*, 2004). Many structural elements are conserved among peroxidases of all three classes leading to the definition of a 'core' peroxidase fold. The structures of class III plant peroxidases including HRP C contain three α helices that are additional to this core peroxidase fold (Gajhede *et al.*, 1997). An entire family of class III peroxidases has been investigated from one plant. According to recent studies there are 73 full-length genes for class III peroxidases in the *Arabidopsis* genome (Tognolli *et al.*, 2002). The peroxidase gene family of *Arabidopsis thaliana* has served as a basis for the comprehensive survey of evolutionary relationships among class III plant peroxidases from angiosperms, gymnosperms, ferns, mosses and liverworts (Duroux and Welinder, 2003).

Rapid reactions of the resting peroxidases with peroxides to form Compound I and the presence of hydrogen bonding network on the distal side of heme distinguish peroxidases from other classes of heme proteins such as globins (Tanaka *et al.*, 1998).

1.1.2. Purification of peroxidases from different sources

Over the years peroxidases have been purified from a number of sources. Bach and Chodat for the first time in nineteenth century obtained a crude preparation of HRP. It was only through the efforts of Richard Willstátter (1872-1942) and Hugo Theorell (1903-1982) that the pure enzyme was finally isolated. Purification of the peroxidases involves techniques such as gel filtration, affinity chromatography, ion-exchange chromatography and high pressure liquid chromatography. For example, HRP was purified in the very beginning by the classical method of affinity chromatography (Brattain *et al.*, 1976). Peroxidases have been isolated from tomatoes (Rodriguez-López *et al.*, 2000a). A cationic peroxidase was purified from melon (Rodriguez-López *et al.*, 2000b). A novel peroxidase has been purified from fresh flowers of *Cynara scolymus*, L (Lopez-Molina *et al.*, 2003). A soluble peroxidase from oil palm (*Elaeis guineensis* Jacq.) leaf was purified using ammonium sulphate fractionation, DEAE cellulose column and SG-100 column chromatography (Deepa and Arumughan, 2002). A novel class III peroxidase isoenzyme was also isolated from tea leaves (Kvaratskhelia *et al.*, 1997). An acidic isoperoxidase has also been purified from turnip roots (Duarte-Vazquez *et al.*, 2003a). Various workers have already purified peroxidase isoenzymes from Brussels sprouts (Mc Lellan and Robinson, 1987), pepper fruit (Pomar *et al.*, 1997), okra (Yemenicioglu *et al.*, 1998), potato (Boucoiran *et al.*, 2000) and green peas (Halpin *et al.*, 1989). The purification and characterization of extensin peroxidase has also been reported (Price *et al.*, 2003). Two distinct peroxidase activities in corn root plasma membrane have also been purified (Mika and Lüthje, 2003).

Although a number of peroxidases have been purified so far, none of them have been able to challenge the superiority of HRP in various clinical and biotechnological applications.

1.1.3. Isoenzymes of peroxidases

The peroxidase isoenzymes in higher plants may be extremely high (Mika and Lüthje, 2003). Up to 40 genes corresponding to isoperoxidases for each plant can be

generated by post transcriptional and post translational modifications (Welinder *et al.*, 1996; De Marco *et al.*, 1999). The peroxidase isoenzymes in plants may differ by more than 50% in amino acid sequence (Welinder, 1992b). There are different regulatory means in vivo to shift optimal conditions from one isoenzyme to another and thereby favor different processes (De Marco *et al.*, 1999). The presence of 70 or more isoforms of peroxidases has made it difficult to define a specific physiological function(s) for each gene of peroxidase (Sasaki *et al.*, 2004). Three peroxidase isoenzymes have been reported from broccoli stems (Thongsook and Barrett, 2005). Purification of an isoperoxidase has also been reported from geranium (Lee *et al.*, 2001). Three forms of anionic peroxidase from cotyledons of cucumber have also been purified (Repka, 2000). Two peroxidase isoenzymes from *Aloe barbadensis* outer mesophyll leaf cells have also been purified (Esteban-Carrasco *et al.*, 2002). Each plant species contains a 'suite' of peroxidase isoenzymes with the potential to carry out a range of different functions (Veitch, 2004). Five peroxidase isoenzymes from tomato roots representing the majority of the total activity in this organ have been purified (Quiroga *et al.*, 2000). Recently the reactions catalyzed by peroxidases have been analyzed in depth at both the structural and the mechanistic level, with new insights obtained from site-directed mutagenesis (Smith and Veitch, 1998). However, the information available does not explain the occurrence and physiological significance of the multiple peroxidase isoenzymes. Post-transcriptional modification may contribute to the number of peroxidase isoenzymes (van den Berg and Wijsman, 1981). Various anionic and cationic isoforms of peroxidases have been found in all plant species studied (Boeuf *et al.*, 2000). Ascorbate peroxidase isoenzymes are distributed in at least four distinct cell compartments; the stroma, thylakoid membrane in chloroplasts, the microbody and the cytosol (Asada, 1992; Miyake and Asada, 1992; Ishikawa *et al.*, 1998). The precise role of individual isoforms of peroxidases remains unclear owing to the lack of information on the localization of the enzymes and the availability of their specific substrates in vivo (Brownleader *et al.*, 1995). However, it has been reported by some workers that there is an abundance of peroxidase isoforms in the cell walls of plants, for example, 47 electrophoretically-separable forms are found in tobacco tissues (Kay and Basil, 1987). Three new 'wall-bound' peroxidase isoenzymes have been reported from tomato fruit (Andrews *et al.*, 2002).

Isoenzymes of peroxidases have been studied in detail and it has been found that cationic peroxidases are involved in auxin metabolism whereas anionic peroxidases are thought to participate in lignification (Mehlhorn *et al.*, 1996). A comparison of the amino acid sequences of peroxidases showed that multiple isoforms with a high sequence similarity respond to stress in different or similar ways (Sasaki *et al.*, 2004). Multiple peroxidases function differently or cooperatively in the same physiological reactions indicating the reasons for the existence of a large number of peroxidase isoenzymes in a single plant species (Hiraga *et al.*, 2000). Peroxidase isoenzymes could also be specifically distributed and there is a possibility that these isoenzymes are expressed by distinct regulatory mechanisms. It is likely that the expression patterns of some peroxidase isoenzymes e.g. ascorbate peroxidase are individually regulated at each cellular compartment under several stress conditions and that each expressed isoenzyme plays a cooperative role to protect each organelle and minimize tissue injury. (Yoshimura *et al.*, 2000).

1.1.4. Substrates of peroxidases

Peroxidases can catalyze the oxidation of a large variety of substrates which range from a protein, cytochrome C, as in the case of cytochrome C peroxidase, to small aromatic molecules for plant peroxidases, to large polymers, as lignin and inorganic ions as Mn^{2+} in the case of lignolytic fungal peroxidases (Banci, 1997). Guaiacol (2-methoxy phenol) is commonly used as a reducing substrate by the class III peroxidases (Kvaratskhelia *et al.*, 1997). Class III peroxidases are known to preferentially oxidize organic phenolic compounds and exhibit a poor activity for ascorbate (Chen and Asada, 1989). Tea peroxidase is the first example of class III peroxidase with a remarkably high activity towards ascorbate (Kvaratskhelia *et al.*, 1997). Class III peroxidases can oxidize a variety of physiological substrates and in general demonstrate little substrate specificity (Price *et al.*, 2003). The low substrate specificity of peroxidases with aromatic substrates can be related to the wide open substrate access channel (SAC) and the absence of substrate specific docking sites (Price *et al.*, 2003).

Crystallographic studies of HRP C-ferulic acid complex demonstrated that this natural aromatic substrate, ferulic acid binds proximal to the exposed heme C_{18}-

methyl, where it forms hydrogen bonds with the catalytic Arg-38 (Henriksen *et al.*, 1999). The coordination of small aromatic substrates to this site within the open SAC appears to be mediated by hydrophobic interactions of the substrate with hydrophobic residues of the channel (Price *et al.*, 2003). All known peroxidase structures display an equatorial cleft that traverses the SAC and this topographical feature is important for the orientation and closer approximation of macromolecular substrates to the active site (Price *et al.*, 2003).

Peroxidases catalyze the reduction of H_2O_2 by taking electrons to various donor molecules. In case of class III plant peroxidases, the donor molecules can be phenolic compound, lignin precursors or secondary metabolites (Passardi *et al.*, 2004). Plant peroxidases can also oxidize the growth hormone, auxin (Gasper *et al.*, 1982). The distal histidine in plant peroxidases is necessary for catalytic activity (Passardi *et al.*, 2004). Guaiacol oxidation in the presence of H_2O_2 is a common method used to detect class III peroxidase activity (Greppin *et al.*, 1986). Earlier workers have characterized plant peroxidases whose catalytic activity was not influenced by Ca^{++} or Mn^{++} ions (Loukili *et al.*, 1999).

A number of structural features contribute to the catalytic activity and efficiency of peroxidases. These include coordination and spin-state of heme iron, the amino acid environment around heme, the hydrogen-bonding network, the electrostatic charge distribution in the heme cavity and the accessibility of heme to hydrogen peroxide and reducing substrates (Kamal and Behere, 2003). The specificity towards different substrates depends on several factors such as the covalent and electrostatic factors as well as the structural properties of the peroxidase itself (Banci, 1997). The protein structure can determine specific binding sites for substrates and mediators or sites for the interaction with bulky substrates. This specificity is probably modulated by changes in the surface topography and in a small number of amino acid substitutions, without any significant change in the folding and in the arrangement of the secondary structure elements (Li and Poulos, 1994; Poulos *et al.*, 1995). The formation of stable complexes between a variety of aromatic donor molecules including ferrulic acid and the cyanide adduct of HRP has already been studied extensively by NMR techniques (Veitch, 1995). Some workers have also proposed a general mechanism for the oxidation of small phenolic substrates (Henriksen *et al.*, 1999).

1.1.5. Glycosylation of peroxidases

Glycans are important for the stability of proteins in general (Faye *et al.*, 1989). The association of peroxidase and glycans has been known for a long time (Olden *et al.*, 1985; van Huystee, 1987). The carbohydrate moieties of the glycoproteins are usually heterogeneous with a number of individual units, as well as various degrees of branching and distribution in the polypeptide chain. The sugars usually form oligosaccharide chains N-linked to asparagine residues of the protein such as found in peroxidase from horseradish (Welinder, 1985) and peanut (Hu and van Huystee, 1989). HRP has been known to be glycosylated since many years (Shannon *et al.*, 1966). A preliminary analysis of the sugars from some of the glycans of HRP was carried out in 1976 (Clarke and Shannon, 1976). The total carbohydrate content of HRP C is somewhat dependent on the source of the enzyme and values between 18-22% are typical (Vietch, 2004). A more detailed analysis of the sugars of SBP was carried out much later (Gray *et al.*, 1996). SBP is glycosylated corresponding to approximately 18% of the molecular mass and the glycans are known to be heterogeneous (Gray *et al.*, 1996; Gray and Montgomery, 1997).

Glycoscience as a whole holds great promise (Fussenegger *et al.*, 1999; Koeller and Wong, 2000; Alper, 2001). Glycans have become increasingly important in diseases such as AIDS (Feizi and Larkin, 1990). Glycosylation plays an important role in secretion (Fiedler and Simons, 1995). It also confers specificity to hormones (Hartree and Renwick, 1992) and provides specific recognition sites (Gahmberg and Tolvanen, 1996). Many pathways in developing plants depend on various glycosylated enzymes (Kermode, 1996; Ishizaki *et al.*, 2002).

The specific glycan structure and their attachment site to the protein is important (Joao and Dwek, 1993; Kimura *et al.*, 1998). Glycans greatly influence the activity of the enzyme; the position of the glycans in relation to the active site was also important (van Huystee *et al.*, 2002). The enzymatic activity of TP was decreased after carbohydrate removal (Duarte-Vazquez *et al.*, 2003b). It has been shown that individual N-linked glycans from a cationic peanut peroxidase contribute towards its function (Lige *et al.*, 2001). All the three-oligosaccharide chains were required for a stable conformation of the protein but only two were necessary for expression of the

catalytic activity. These findings support the hypothesis that each glycosylation site may involve a distinct function in the overall behavior of enzyme glycoproteins.

It has been demonstrated that glycans may have an important role in maintaining the stability of peroxidases as in cationic peanut peroxidase (Hu and van Huystee, 1989). It was much later deduced that glycans of cationic peanut peroxidase showed an important role in secretion (van Huystee *et al.*, 2002). In cationic peanut peroxidase, it has been reported that glycans provide resistance against trypsin digestion (Lige *et al.*, 2001). In a study of five different glycoproteins analyzed on the basis of stability, the role of glycans was found to be related to the folding and subsequent stability of the proteins (Wang *et al.*, 1996). The most important general effect of glycosylation is to increase solubility and reduce aggregation of the proteins (Tams *et al.*, 1999). Glycosylation also helps in maintaining the storage stability of a protein (Sánchez-Romero *et al.*, 1994; Nie *et al.*, 1999). Tigier *et al.* (1991) have described that carbohydrate moiety in a protein normally affects enzyme kinetics. Removal of carbohydrate moiety has a profound effect on antigenicity and resistance to protein attack (Hu and van Huystee, 1989). The carbohydrate moiety blocks the access of proteases to the potential proteolytic cleavage sites or stabilizes the conformation of the domain where the cleavage sites are exposed (Schwarz and Datema, 1982). In addition the carbohydrate removal produces protein conformational changes exposing amino acid sequences recognized by proteases and eventually the protein is destroyed (Barriocanal *et al.*, 1986). Partial carbohydrate removal produced an unstable avocado peroxidase (Sánchez-Romero *et al.*, 1994). Deglycosylation studies have been performed on peroxidases from peanut (Hu and van Huystee, 1989), seeds of peach fruit (Tigier *et al.*, 1991), and horseradish (Tams *et al,* 1999).

Several investigators have demonstrated that removal of carbohydrate moiety affected various physical and biological properties of proteins (Tigier *et al.*, 1991; Nie *et al.*, 1999). N-linked glycan structures influence protein targeting, stability and function (Lerouge *et al.*, 1998; van Huystee and McManus, 1998). Carbohydrate moieties of turnip peroxidase did not influence the conformation of the polypeptide backbone. However, its presence considerably enhanced conformational stability of proteins towards heat (Duarte-Vazquez *et al.*, 2003b). This was probably due to hydrogen bonding with polypeptide backbone (Wang *et al.*, 1996). Studies on glucose oxidase crystal structure, where N-linked mannose residues formed strong hydrogen

bonds with the carbonyl oxygen of glutamic acid, are further evidence to this suggestion (Hecht *et al.*, 1993). There were few comparative studies that directly address the role of N-linked glycan structures attached to peroxidase. Some workers have used the approach of site directed mutagenesis to remove N-linked sites individually and to study the resulting change in enzyme stability. For example, the N-linked sited were removed in the case of cationic peroxidase of peanut (Lige *et al.*, 2001). Purification of the mutated proteins from a transgenic tobacco leaf revealed that ablation of glycosylation at some specific sites exhibited a profound influence on protein function, specific activity, thermal stability or protein folding depending on the specificity and number of the glycosylation sites ablated (Lige *et al.*, 2001).

In terms of plant peroxidases, the published evidence suggests a role for the N-linked glycan structures. It has been shown that complete removal of N-linked glycans resulted in the decrease of thermostability of peanut peroxidase (Pathirana *et al.*, 2005). Partial or complete deglycosylation of peroxidases has been shown to influence the affinity of the enzyme for various substrates. Deglycosylation also influences the relative accumulation of mutated peanut peroxidase in cell wall as compared to wild type peanut peroxidase (Pathirana *et al.*, 2005).

1.2. ROLE OF PEROXIDASES IN PLANTS

The diversity of reactions catalyzed by plant peroxidases explains their implications in a broad range of physiological processes. It is unlikely that a single peroxidase will catalyze a single specific reaction in vivo (Andrews *et al.*, 2002). This was also because of high redundancy found in peroxidase genes (Christensen *et al.*, 1998). The in vivo function of a peroxidase is likely to be determined by its location in a particular tissue, cellular or sub-cellular compartment, which in turn, may depend in part on its ionic nature (Carpin *et al.*, 2001).

It has been reported that peroxidases were involved in various metabolic steps such as auxin catabolism (Normanly *et al.*, 1995). They catalyzed crosslinking in cell walls by formation of diferuloyl bridges between pectin residues and isodityrosine bridges between hydroxyproline rich extension molecules (Schnabelrauch *et al.*, 1996; Brownleader *et al.*, 1999; Hatfield *et al.*, 1999; Amaya *et al.*, 1999). It was observed that the enzymes were also involved in the oxidation of cinnamyl alcohols

prior to their polymerization during lignin and suberin formation (Whetten *et al.*, 1998). The oxidation of syringaldazine, a lignin monomer analog, by a peroxidase was suggested to be indicative of its involvement in the synthesis of lignin and suberin (Christensen *et al.*, 1998). Other studies have used the expression for specific peroxidase genes in lignifying or suberizing plant tissues as the argument for their role in these biosynthetic pathways (Mohan *et al.*, 1993). Peroxidases are required for lignin synthesis but they also occur in cell walls that do not form lignin (Brownleader *et al.*, 1995). Cationic peroxidases have been found to be most effective in IAA catabolism and anionic isoforms are believed to be associated with lignification (Brownleader *et al.*, 1995).

The cessation of fruit growth in plants is related to a developmental increase in peroxidase activity associated with exocarp (Andrews *et al.*, 2002). There is a peroxidase mediated stiffening of exocarp cell walls leading to the cessation of fruit growth. However, anionic peroxidase isoenzymes play a major role in fruit development by localizing within the cell walls of the exocarp. Thus, the peroxidase isoenzymes have a dual role in restricting fruit expansion through crosslinking of cell wall components and producing a protective barrier in the epidermis (Andrews *et al.*, 2002).

Extensin is a structural protein belonging to the family of hydroxyproline rich glycoproteins and a major component of dicot cell walls (Price *et al.*, 2003). The formation of extensin network in higher plants requires peroxide and peroxidase. Capacity to efficiently crosslink extensin is not a property of class III peroxidases in general but is limited to particular peroxidases, referred to as extensin peroxidases (EPs) (Jackson *et al.*, 1999; Jackson *et al.*, 2001). EPs exhibit an important role in defense against fungal infection and UV irradiation (Price *et al.*, 2003).

Peroxidases have been associated with an ever-increasing number of physiological processes. These include leaf and flower abscission, aging and senescence, apical dominance, cold tolerance, dormancy, fruit development and ripening, germination and early development (Mehlhorn *et al.*, 1996). Several investigators have reported a role of peroxidases in defense against pathogens (Brownleader *et al.*, 1995; Kawalleck *et al.*, 1995; Fossdal *et al.*, 2001). Possible functions of peroxidases like detoxification or production of reactive oxygen species as signal mediators or anti microbial agents at the interface of cell wall/plasma

membrane could be part of defense mechanisms against pathogen infection (Hiraga *et al.*, 2001). Peroxidases that catalyze the crosslinking reactions may be expressed in response to external factors such as the wounding of plant tissue. Water loss and invasion by pathogens can thus be checked by peroxidases by the formation of a protective polymeric barrier such as suberin (Veitch, 2004). Studies on the expression of an anionic peroxidase in transgenic tobacco plants indicate that while overproduction of the enzyme favors defense strategies such as resistance to disease, physical damage and insect attack. It has a negative impact on growth because of increased indole acetic acid (IAA) degradation activity (Lagrimini, 1996). Thus peroxidase expression in plant tissues at different stages of development must reflect a balance between the priorities of defense and growth. It has been shown that wound response and pathogen attack activates peroxidase genes indicating their role in restoration of damaged tissue and defense against pathogen attack (Chittoor *et al.*, 1997; Hiraga *et al.*, 2000). There was increasing information available from microarray analysis of selected genes in relation to plant defense in peroxidases (Schenk *et al.*, 2000). It is important to note the distinction between the peroxidases of the pathogen and those of the host plant and that the pathogen peroxidases may contribute towards the increased activities of total peroxidases in disease resistance (Brownleader *et al.*, 1995). The response to injury consists of the production of H_2O_2 and other reactive oxygen species that are toxic. The oxidation of phenolic compounds by peroxidases in the presence of H_2O_2 firstly produces more toxic species such as phenoxy radicals and quinones and finally polymers, tannin or melanin type which can protect against injury by their toxic properties and by sealing the affected area (Diehn *et al.*, 1993; Esteban-Carrasco *et al.*, 2001). Peroxidases exhibit a major role in defense of the plant against herbivores (Gutterman and Chauser-Volfson, 2000).

A flavanoid-peroxidase catalyzed reaction product has proved its potential in H_2O_2 scavenging (Yamasaki *et al.*, 1997). Some class III plant peroxidases and ascorbate peroxidase (class I peroxidase) are involved in H_2O_2 scavenging systems (Mehlhorn *et al.*, 1996; Kvaratskhelia *et al.*, 1997). Hiraga *et al.* (2000) have described that UV-inducible peroxidase genes are involved in the detoxification of H_2O_2, which accumulates as a result of UV-irradiation, exposure to air pollution or high intensity light.

Plants are able to cope with the effects of overproduction of partly reduced oxygen species during oxidation stress by regulating antioxidant enzymes such as peroxidases (Gorecka *et al.*, 2005). Peroxidases are also involved in the formation of activated oxygen species that are in turn involved in the modulation of mechanical properties of cell wall (Rodriguez *et al.*, 2002; Liszkay *et al.*, 2003). Extensin peroxidase in tomato has been shown to be involved in the formation of rigid wall of cell expansion and plant growth (Brownleader *et al.*, 2000). Many plant peroxidases have been shown to be involved in the responses of plants to a wide range of biotic and abiotic stresses (Castillo, *et al.*, 1992).

1.3. INDUSTRIAL, BIOTECHNOLOGICAL, ANALYTICAL AND MEDICAL APPLICATIONS OF PEROXIDASES

Plant peroxidases are receiving increasing attention due to their extensive bioactivation property and potential applications in clinical, biochemical, biotechnological and related areas (Azevedo *et al.*, 2003). These enzymes are playing a significant role in the remediation of wide spectrum of chemical compounds (Table 1).

1.3.1. Peroxidases in dye decolorization and removal

Dyes are extensively used in a number of industries such as paper printing, color photography, textile industry, and as an additive in petroleum products. Total world colorant production is estimated to be about 800,000 tons/year. About 10-15% of the synthetic dyes produced are discharged into industrial effluents causing environmental pollution (Spadaro *et al.*, 1992; Robinson *et al.*, 2001). The dyes used in textile industries are designed to resist fading upon exposure to light, microbial attack etc, hence they are difficult to degrade. Azo reactive dyes, the largest class of water-soluble synthetic dyes with the greatest variety of colors and structures, are generally resistant to aerobic biodegradation. During the dyeing process most of the dyes are hydrolyzed and later released into waterways. The release of dyes thus

Table 1: Remediation of chemical compounds by using peroxidases

Peroxidase/source	Substrates	References
LiP (White rot fungi)	Polyaromatic hydrocarbons: anthracene, pyrene, benzopyrene.	Wang *et al.*, 2003
	Substituted phenols and 1,2 dimethoxyarenes.	Ward *et al.*, 2003
	Non-phenolic trimeric lignin model compounds e.g. β-o-aryl.	Baciocchi *et al.*, 2003
	Aromatic tertiary amines N-methylcarbazole.	Baciocchi *et al.*, 2002
	Reactive Blue-5	Chairattanamanokorn *et al.*, 2006
	Methoxybenzenes	Kersten *et al.*, 1990
	Synthetic azo dyes (Orange II)	Chivukula *et al.*, 1995
MnP (*Panus tigrinus*) (*Irpes lacteus*) (*Bjerkandera adusta*, *Pleurotus eryngii*)	Aromatic amines, hydrocarbons.	Lisov *et al.*, 2003
	Reactive Blue-5	Chairattanamanokorn *et al.*, 2006
	Azo and phthalocyanine dyes.	Heinfling *et al.*, 1998
Chloroperoxidase (*Caldariomyces fumago*)	4,6-dimethyldibenzo-thiophene (dimer).	Torres and Aburto, 2005
	Halophenols	Osborne *et al.*, 2006
HRP (*Armoracia rusticana*)	Phenol and aniline derivatives.	Gilabert *et al.*, 2005
	o-methoxyphenolic compounds.	Antoniotti *et al.*, 2004
	2,4 dichlorophenol	Laurenti *et al.*, 2003
	Anthracyclines	Reszka *et al.*, 2005
	Dihydroxyphenoxazine derivatives.	Towne *et al.*, 2004
	Phenol, *o*-chlorophenol, 2,4,6-trichlorophenol.	Davidenko, 2004
	o-phenylenediamine	Alpeeva *et al.*, 2003
	Cyclometalated ruthenium (II) complexes.	Fang and Barcelona, 2003

	Aromatic hydrocarbons	
	such as *o*-xylene-d 10 and naphthalene-d8	Xu *et al.*, 2005
	1-naphthol	Bhandari and Xu, 2001
	Methylmethacrylate.	
	N-alkyl-N-phenylglycines.	Totah and Hanzlik, 2004
	Phenols from petroleum	
	Refinery.	Wagner and Nicell, 2003
	Phenolic compounds	
	4-oxyphenol	Cheng *et al.*, 2006
	4-fluorophenol	Shutava *et al.*, 2004
	4-bromophenol	Osman *et al.*, 1997
	Bisphenol F consisting of 2,4' and 4,4'-dihydroxy	
	Diphenylphenylmethanes.	Uyama *et al.*, 2002
	m-ethynylphenol	Tonami *et al.*, 2000
	Chlorpromazine	Goodwin *et al.*, 1997
	Aminopyrine	Goodwin *et al.*, 1996
	Ethylhydroperoxide, 5-phenyl-4-penten-1-yl hydroperoxide (PPHP).	Samokyszym *et al.*, 1995
	Thioanisole and its p-methyl, *p*-methoxy and *p*-nitro analogues.	Harris *et al.*, 1993
	3-alkylindoles	Ling and Sayre, 2005
	Orange II (azo dye)	Kim *et al.*, 2005
	Acid Black 10 BX	Mohan *et al.*, 2005
	Azo dyes (RBBR)	Bhunia *et al.*, 2001
	Phenols	Bewtra *et al.*, 1995
	Estrogens: Estrogens-estrone (E1), 17 β-estradiol (E2), estriol (E3), 17 α-ethinylestradiol (EE2).	Auriol *et al.*, 2006
	2,4,6-trinitrotoluene (TNT)	Beom *et al.*, 2003

Fungal peroxidase (*Coprinus cinereus*)	Chlorophenols Naphthols, 4-hydroxybiphenyl. N-substituted phenothiazines and phenoxazines.	Pezotti *et al.*, 2004 Bratkovskaja *et al.*, 2004 Kulys *et al.*, 2000
Fungal peroxidase (*Coprinus macrorhizus*)	Aromatic compounds	Al Kassim *et al.*, 1994
Catalase peroxidase (*Thermoalkaliphilic Bacillus* sp.)	Textile bleaching effluents	Gudelj *et al.*, 2001
BGP (*Momordica charantia*)	Phenols, phenolic mixtures dyes, mixture of textile dyes, dyeing effluent.	Akhtar and Husain, 2006 Akhtar *et al.*, 2005b Akhtar *et al.*, 2005c
TP (*Brassica rapa*)	Naphthol Phenols Acid dyes	Singh and Singh, 2002 Singh *et al.*, 2000 Duarte-Vazquez *et al.*, 2002 Kulshrestha and Husain, 2006
SBP (*Glycine max*)	Phenols in wastewater. Nitroaromatics	Bodalo *et al.*, 2006; Caza *et al.*, 1999 Mantha *et al.*, 2002

causes an ecotoxic hazard and eventually leads to bioaccumulation that may be transported through the food chain.

In humans, dyestuffs have been found to cause allergic reactions such as eczema or contact dermatitis (Mathelier-Fusade *et al.*, 1996; Khanna and Sasseville, 2001). Reduction of azo dyes, cleavage of dye's azo linkages leads to the formation of aromatic amines. These aromatic amines are known mutagens and carcinogens (Spadaro *et al.*, 1992; Chung *et al.*, 1993).

A number of physical, chemical and biological techniques have been employed for the decolorization of dyes. However, due to certain inherent limitations, these processes alone are incapable of complete remediation of dyes. Attempts have been made to employ a combination of these methods such as chemico-biological, chemico-physical and physico-biological (Shu and Chang, 2005). Currently employed physico-chemical methods for dye decolorization and degradation are chemical oxidation, reverse osmosis, coagulation-flocculation, filtration, adsorption, photodegradation etc (Young and Yu, 1997; O'Neill *et al.*, 1999). These methods have serious limitations such as high cost, high salt content in the effluent and problems related to the disposal of concentrate. Ozonation, coagulation, flocculation and electrochemical methods have resulted in poor color removal. Chemical coagulation and reverse osmosis resulted in a large proportion of the color being removed but the latter was not cost effective. Many bacteria and fungi have been used for the development of biological processes for the treatment of textile effluents (Mielgo *et al.*, 2001; Bhatt *et al.*, 2005). Synthetic dyes contain various substituents such as nitro and sulphonyl groups. Thus, these dyes are not uniformly susceptible to decomposition by activated sludge in a conventional aerobic process. Attempts to develop aerobic bacterial strains for dye decolorization often resulted in a specific strain which was restricted in acting only on a specified dye structure (Kulla, 1981).

Recently, enzymatic approach has attracted much interest in the decolorization/degradation of textile and other industrially important dyes. Enzymatic treatment is very useful due to the action of enzymes on pollutants even when they are present in very dilute solutions and recalcitrant to the action of various microbes participating in the degradation of dyes. The potential of the enzymes; peroxidases, manganese peroxidases, lignin peroxidases has been exploited in the decolorization and degradation of dyes.

Some of the dyes are recalcitrant to the action of these enzymes. However, the addition of certain redox mediators enhanced the range of substrates and efficiency of degradation of the recalcitrant compounds. Several redox mediators have been reported in literature but few of them e.g. 1-hydroxybenzotriazole, veratryl alcohol, violuric acid, 2-methoxy-phenothiazone have been frequently used (Husain, 2006).

It has also been shown that HRP can be effective in degrading and precipitating industrially important azo dyes such as Remazol Brilliant Blue (Bhunia *et al.*, 2001). This enzyme exhibited broad substrate specificity towards a variety of azo dyes. Purified peroxidase of *Saccharum spontaneum* leaf could completely degrade Supranol Green and Procion Green HE-4BD (100%) dyes within one hour, whereas Direct Blue, Procion Brilliant Blue H-7G and Chrysoidine were degraded more than 70% in one hour. Peroxidases from plant, *Ipomea palmate* were also used for the decolorization of eight textile dyes, mainly azo dyes and seven other dyes (Shaffiqu *et al.*, 2002). Purified peroxidase from *Senna angustifolia* catalyzed the oxidation of alizarin and purpurin anthraquinones to the corresponding 3,3'-bializarin and the new compound 3,3'-bipurpurin, respectively, as well as the formation of 2,2'-biquinizarin from quinizarin anthracenone (Arrieta *et al.*, 2002).

Akhtar *et al.* (2005c) have investigated the potential of peroxidase from *Momordica charantia* in decolorizing industrially important dyes. *Momordica charantia* peroxidase was able to decolorize most of the textile dyes by forming an insoluble precipitate. The greater fraction of the color was removed when the textile dyes were treated with increasing concentrations of enzyme. Step-wise addition of enzyme to the decolorization mixture at the interval of one hour enhanced the rate of dye decolorization. Decolorization of non-textile dyes resulted in the degradation and removal of dyes from the solution without any precipitate formation.

Several limitations prevent the use of free peroxidases in dye decolorization. Some of these limitations have been overcome by the use of enzymes in the immobilized form. Immobilized enzymes have greater stability and can be used for a longer period of time as catalysts due to reusability. This greatly reduces the cost in industrial processes (Akhtar *et al.*, 2005a; Khan *et al.*, 2005; Kulshrestha and Husain, 2006). The removal of acid azo dye, Acid Black 10 BX by free and immobilized enzyme has been investigated (Mohan *et al.*, 2005). The immobilized HRP was more effective in removing the high percent of color from synthetic water as compared to

free HRP. Bitter gourd peroxidase immobilized on a bioaffinity support, Concanavalin A (Con A) Sephadex; was highly effective in decolorizing industrially important dyes from polluted water compared to its soluble counterpart. Immobilized enzyme was repeatedly exploited for the decolorization of eight reactive dyes from the fresh batches of dye solutions and after the tenth repeated use, it retained nearly 50% of the initial activity. TOC content of soluble and immobilized BGP treated individual dyes, mixture of dyes and dyeing effluent was determined and it was noticed that higher TOC was removed after treatment with immobilized BGP (Akhtar *et al.*, 2005b)

Lignin peroxidase (LiP) and Manganese peroxidase (MnP) have been shown to be involved in the decolorization of synthetic azo dyes such as Orange II (Thurston, 1994; Chivukula *et al.*, 1995). Studies on the enzymatic degradation of Remazol Brilliant Blue R (RBBR) showed that partially purified LiP might have a role in the decolorization of this dye (Young and Yu, 1997). MnP from *Bjerkandera adusta* and *Pleurotus eryngii* could transform six industrially important azo and phthalocyanine dyes. LiP from *B. adusta* showed low activity with most of the dyes but the specific activities increased 8-100 folds when veratryl alcohol was included in the reaction mixture. MnP isoenzymes could efficiently decolorize the azo dyes and the phthalocyanine complexes in a Mn^{2+} -independent manner (Heinfling *et al.*, 1998). Moreira *et al.* (2001) have evaluated the enzymatic action of the ligninolytic enzyme MnP, through a suitable addition of H_2O_2, as a feasible system for the *in vitro* degradation of complex structures. The highly recalcitrant polymeric dye Poly R-478 was selected as a model compound.

The oxidation of various recalcitrant xenobiotics released in the environment was carried out by LiP, MnP, other oxidases and laccases (Hatakka *et al.*, 2003). An extracellular peroxidase produced by *Pleurotus ostreatus*, an edible macroscopic fungus can decolorize RBBR (Shin *et al.*, 1997). Peroxidase from *Pleurotus ostreatus* was also effective in the decolorization of dyes belonging to four structurally different groups, triaryl methane, heterocyclic, azo and polymeric (Shin and Kim, 1998).

1.3.2. Peroxidases in phenol removal

Aromatic compounds such as phenols and its derivatives are a major class of pollutants in wastewater from a number of food and chemical industries (Nicell *et al.*, 1993; Duran and Esposito, 2000; Husain and Jan, 2000). Phenols are known to be toxic and also hazardous carcinogens that can accumulate in the food chain. Due to their toxicity, phenols must be removed from wastewater prior to their final discharge in the environment (Karam and Nicell, 1997).

In recent years a great deal of research has been directed towards developing processes in which peroxidases are used to remove phenolic contaminants from toxic/polluted wastewater (Duran and Esposito, 2000; Husain and Jan, 2000; Torres *et al.*, 2003). The removal of phenols by peroxidases from wastewater is now well documented and peroxidases from different sources are currently being employed for the removal of phenols (Duarte-Vazquez *et al.*, 2002; Wagner and Nicell, 2003). HRP has been widely used in the removal of phenols (Bewtra *et al.*, 1995). Peroxidases from other sources were also found to be effective in phenol removal for example SBP (Caza *et al.*, 1999; Kinsley and Nicell, 2000; Kennedy *et al.*, 2002), TP (Duarte-Vazquez *et al.*, 2002; Duarte-Vazquez *et al.*, 2003b) and BGP (Akhtar and Husain, 2006).

Phenols are oxidized by peroxidases to generate phenoxy radicals, which couple to form oligomeric and polymeric products (Husain and Jan, 2000; Ward *et al.*, 2001). These polymeric products have limited water solubility and tend to precipitate quite readily. The precipitation of the product is thought to be accompanied by removal of phenols and other related aromatic compounds from wastewater. The insoluble products could be simply removed by centrifugation, sedimentation or filtration (Klibanov *et al.*, 1983).

The polymerization reaction can be written as follows:

$H_2O_2 + 2AH_2 \rightarrow 2H_2O$ + polymerized products (HAAH)

HAAH can serve as a hydrogen donor, leading to higher degrees of polymerization. Enzyme treatment offers a high degree of specificity, operation under mild conditions and high reaction velocity (Karam and Nicell, 1997).

Enzyme immobilization is one of the strategies known to increase enzyme stability and reusability. It also enables continuous conversion process (Duran and Esposito, 2000; Duran *et al.*, 2002). Soluble and immobilized BGP have been used for the removal of phenols such as parent phenol, *p*-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol. Most of the phenols studied were removed over the wide pH range 4.0-8.0. Both soluble and immobilized BGP were capable of removing phenols maximally at pH 6.0. However, the immobilized BGP could remove a higher percentage of phenols over a wide range of pH as compared to soluble BGP. Maximum removal of phenol and 2,6-dichlorophenol was observed at 40 °C. Immobilized BGP was capable of removing higher concentration of phenol and 2,6-dichlorophenol at various temperatures (Akhtar and Husain, 2006). A broad range of pH-optimum for the removal of phenols from aqueous solutions has also been reported for many peroxidases (Duarte-Vazquez *et al.*, 2002). Maximum phenol removal at pH 6.0 has been obtained by using SBP (Caza *et al.*, 1999) and TP (Duarte-Vazquez *et al.*, 2001). Duarte-Vazquez *et al.* (2003b) studied the efficiency of TP to remove different phenolic compounds such as phenol, *p*-chlorophenol, o-cresol, *m*-cresol and 2,4-dichlorophenol and bisphenols from polluted water.

Immobilized BGP treated phenolic mixtures exhibited significant loss of total organic carbon (TOC) from solutions (Akhtar and Husain, 2006). Immobilized peroxidase was also able to remove a high percentage of TOC from model wastewater containing mixture of phenols (Tatsumi *et al.*, 1996; Akhtar and Husain, 2006).

Colored products were formed during the conversion of phenols by peroxidases. This stayed in the aqueous solution and created a major hindrance (Husain and Jan, 2000). This problem can be solved by the application of a coagulant after an enzymatic reaction (Tonegawa *et al*, 2003). Coagulants were effective in protecting enzymes against inactivation induced by the reaction product (Tatsumi *et al.*, 1994; Duran and Esposito, 2000; Husain and Jan, 2000). Chitosan, a derivative of natural polymer chitin, is considered as a good absorbent for phenoxy radicals originating from the oxidation of phenols (Tatsumi *et al.*, 1994). The application of activated carbon appeared to be an inexpensive and efficient approach for color removal from peroxidase treated phenols. A maximum color removal was achieved within a relatively short time of 2 h after carbon addition (Tonegawa *et al.*, 2003).

Activated DEAE cellulose-11 has also been used as an adsorbent for the removal of soluble colored product formed by the action of BGP (Akhtar and Husain, 2006).

Peroxidases have the ability to co-precipitate certain difficult to remove contaminants including non-substrates of peroxidases along with the more easily removable compounds by inducing the formation of mixed polymers. These polymers behaved similarly to the polymeric products of easily removable compounds (Duran and Esposito, 2000). This is of practical importance since many industrial effluents contain a variety of phenolic contaminants; some of which are more amenable to enzymatic treatment than others. The potential of peroxidase for soil and water detoxification constitute a possible basis for the development of remediation technologies. For example, the herbicide atrazine, was biotransformed to the less toxic compounds such as hydroxylatrazine. The activity production of lithium peroxidase and MnP coincided with the degradation of atrazine (Mougin *et al.*, 1994). A soil sample containing a triazine was biodegraded by a bacterial strain. Fournier et al. (2004) have shown that MnP was responsible for its biodegradation

1.3.3. Peroxidases in organic and polymer synthesis

Peroxidase catalysis produces free radicals, which participate in different post-enzymatic reactions. Oxidative polymerization of aromatic compounds mediated by oxidoreductases has been employed to create new functional polymers and to synthesize phenolic resins with good chemoselective conversion (Regalado *et al.*, 2004). HRP has been used to polymerize phenolic and aromatic amine compounds while new types of aromatic polymers have been synthesized in water and water-miscible organic solvents (Oguchi *et al.*, 1999). Cardanol is a phenol derivative having a C15 unsaturated alkyl chain with 1 to 3 double bonds at its meta position (Ikeda *et al.*, 1998). It has been reported that SBP catalyzed the oxidative polymerization of cardanol (Kim *et al.*, 2003).

The production of conducting polymers has remarkable interest because of their wide range of applications, including anticorrosive protection, optical display, light emitting diodes etc. (Raitman *et al.*, 2002). Polyaniline is one of the most extensively investigated conducting polymers. HRP has been used in the synthesis of this polyaniline (Lui *et al.*, 1999). Recently some workers have used anionic peroxidase purified from African oil palm tree as biocatalysts and developed an

enzymatic synthesis of polyelectrolyte complex of polyaniline and sulfonated polystyrene (Sakharov *et al.*, 2003). Enzymatic polymerization of substituted and unsubstantiated phenols and anilines was catalyzed by HRP using a template, which could be a micelle, a boxate containing electrolyte or lignin sulfonate (Nagarajan *et al.*, 2003). In presence of H_2O_2 a peroxidase catalyzes the oxidation of phenols that eventually gives rise to higher molecular weight (M_r) polymers (Nicell and Wright, 1997). Some workers have successfully obtained copolymerization of lignin with cresol in a reversed micellar system (Liu *et al.*, 1999).

MnP produced by the basidiomycete *Bjerkandera adusta* was used for acrylamide polymerization in the presence of 2,4-pentanedione as a radical initiator (Iwahara *et al.*, 2000). A patent has been filed in relation to in situ crosslinking of proteins, including collagen by using HRP, which converted collagen into biocompatible semi-solid gel. This gelling material can be used as wound sealant, delivery vehicle or as binding agent in food product applications (Prochaska *et al.*, 2003).

1.3.4. Peroxidases in paper pulp industry

Biopulping is a process where the extracellular enzymes produced by a white rot fungus remains adsorbed on the wood chips, degrading lignin (Brasil de Souza-Cruz *et al.*, 2004). After the pulping process, about 10% of the lignin appears as modified, which is responsible for a characteristic brown color. Modified lignin can be enzymatically degraded using a biobleaching process (Antonopoulos *et al.*, 2001). Major lignin degrading enzymes from basidiomycete like MnP, laccase and LiP are used in this process (Hatakka *et al.*, 2003).

1.3.5. Peroxidases in biosensors

A biosensor is an analytical device which utilizes the sensitivity and selectivity of a bio-receptor attached on to the surface of a physical transducer. The transducer is able to respond to and transform a biochemical and/or physico-chemical property into a measurable signal as a result of a biorecognition event between the bioreceptor and its target analyte (Thevenot *et al.*, 2001; Patel, 2002). In enzyme-based biosensors, the biological element is the enzyme, which reacts selectively with

its substrate (Guilbert *et al.*, 2004). The first biosensor was an enzyme sensor developed by Clark and Lyons (1962). In this biosensor glucose oxidase was attached onto the surface of an amperometric oxygen electrode and was used to directly quantify the amount of glucose in a sample. Since then numerous enzyme sensors in different configurations have emerged. Some biosensors use techniques for toxicity monitoring for environmental, agricultural and food applications. A number of workers have already shown the immobilization of royal palm tree peroxidase and HRP onto graphite electrodes (Brusova *et al.*, 2005). HRP has been extensively used in the construction of biosensors (Ruzgas *et al.*, 1996a). Incorporation of immobilized enzymes in the development of biosensors has come to solve several problems such as the loss of enzyme (especially if expensive), maintenance of enzyme stability and shelf life of the biosensor and reduction in the time of organic response (Amine *et al.*, 2006).

Recently, peroxidase based electrodes have demonstrated the widespread use in analytical systems for the determination of hydrogen peroxide and organic hydroperoxide (Jia *et al.*, 2002). When co-immobilized with a hydrogen peroxide producing enzyme, they can be exploited for determination of glucose, alcohols, glutamate, choline (Ruzgas *et al.*, 1996b). An electrode substitutes the electron donor substrate in a common peroxide reaction cycle; the process is called as direct electron transfer (Freire *et al.*, 2003).

Enzymes immobilized on an electrode can be oxidized by hydrogen peroxide:

$Fe^{III} + H_2O_2 \rightarrow [(Fe^{IV}=0)\text{-}R^{+\cdot}]_{COI} + H_2O$

Where COI stands for compound I

The oxidized enzyme is then reduced by electrons provided by an electrode,

$[Fe^{IV}=0\text{-}R^{+\cdot}]_{COI} + 2e^- + 2H^+ \rightarrow Fe^{II} + H_2O.$

When an electron donor 'A' is present in a peroxidase-electrode system, the direct process can occur simultaneously, with reduction of the oxidized donor 'A^{o}' by the electrode (Liu and Ju, 2002).

During direct electron transfer, electrons act as the second substrate for the enzymatic reaction, resulting in a shift of electrode potential for the enzymatic reaction, with the measured current being proportional to the H_2O_2 concentration (Regalado *et al.*, 2004). This technique can also be used to quantify other metabolites, especially combined with another oxidase. Peroxidase can also interact with an

electrode by mediated electron transfer, where a mediator, an electron donor, 'A' transports the electrons between the enzyme and the electrode. In this system, the enzymatically-oxidized donor 'A^{o}' is electrochemically reduced by the electrode. When H_2O_2 and an aromatic electron donor 'A' are present at a peroxide electrode, both direct and mediated electron transfer can occur simultaneously. This phenomenon has been used to develop a method for determination of phenols and aromatic amines down to the nanomolar range (Monteanu *et al.*, 1998). Some workers have demonstrated the potential of using pyrolytic graphite electrodes made of HRP immobilized in TiO_2 films in electrochemistry and bioelectrocatalysis (Zhang *et al.*, 2004). Earlier some workers had also used graphite-teflon peroxidase composite electrodes for amperometric detection of 18 phenolic compounds (Serra *et al.*, 2001). Mulchandani and Pan (1999) have developed an amperometric biosensor using HRP to measure H_2O_2 and other organic peroxides.

1.3.6. Peroxidases as analytical and diagnostic agents

Peroxidases have a number of practical analytical applications. These enzymes have the ability to yield chromogenic products at low concentrations and they are well suited for the preparation of enzyme conjugated antibodies and application in diagnostic kits (Krell, 1991).

Several investigators have used purified peroxidase isoenzymes from turnip (*Brassica napus*) and horseradish to develop a diagnostic kit for determination of uric acid (Krell, 1991; Agostini *et al.*, 2002). A simple and economical biostrip technology for the estimation of lactose by immobilizing β-galactosidase, galactose oxidase and HRP into a polymeric support has been developed (Sharma *et al.*, 2002). The biostrip was dipped in milk or a milk product and the color developed from an added chromogen was used to estimate lactose. An enzymatic colorimetric method has been developed for total cholesterol determination, which employs cholesterol oxidase, cholesterol esterase and peroxidase immobilized individually resulting in high selectivity and increased stability (Malik and Pundir, 2002). In this method cholesterol ester is hydrolyzed by cholesterol esterase to free fatty acid and

cholesterol, which is oxidized by cholesterol oxidase to colestenone and H_2O_2. H_2O_2 is determined by using HRP.

HRP has also been used in several diagnostic applications in medicine such as the detection of 8-hydroxy deoxyguanosine and its analogs in urine to identify bladder and prostate cancer risk. (Chiou *et al.*, 2003). A solid phase enzyme immunoassay was developed to measure human tumor necrosis factor-alpha in clinical research using bispecific antibodies hTNF-alpha and HRP (Berkova *et al.*, 1996). HRP and the plant hormone indole-3-acetic acid (IAA) have been used in gene-directed therapy. HRP/IAA represented an efficient system for enzyme/prodrug-based anticancer approach (Greco *et al.*, 2001).

1.3.7. Peroxidases in immunoassays

Enzyme linked immunosorbent assay is popularly known as ELISA. These tests are designed to detect antigens or antibodies by producing an enzyme-triggered change of color. HRP is probably the most common enzyme used as a reporter, enzyme labeled antibody in enzyme immunoassays. Mycotoxins are dangerous by-products of several fungi; *Aspergillus*, *Pencillium* and *Fusarium* species. ELISA tests in which peroxidase is used for the labeling of an antibody, have been developed for screening monoclonal antibodies against mycotoxins (Kawamura *et al.*, 1989). ELISA test, employing peroxidase as reporter enzyme has also been developed to detect hepatitis-B virus in human serum (Zhuang *et al.*, 2001). A competitive indirect ELISA using peroxidase as reporter enzyme has been developed for detection of alkaline phosphatase in milk (Vega-Warner *et al.*, 2000).

1.3.8. Peroxidases in cancer therapy

One of the most interesting reactions of HRP C occurs with the plant hormone, indole-3-acetic acid. Many peroxidase-catalyzed reactions take place without hydrogen peroxide, hence the term, indole acetic acid oxidase has been used to describe this activity of HRP C in the past literature. Most recent studies of the reaction at neutral pH indicate that it is not an oxidase mechanism that operates but rather a peroxidase mechanism coupled to a very efficient branched-chain process in

which organic peroxide is formed (Dunford, 1999). A combination of IAA and HRP C can offer a new potential for targeted cancer therapy (Folkes and Wardman, 2001; Greco *et al.*, 2001; Wardman, 2002). These workers also observed that IAA is cytotoxic towards mammalian cells, including human tumor cells in the presence of HRP C.

The primary mechanism of toxicity is thought to involve 3-methylene-2-oxindole, a known product of the reaction between HRP C and IAA that shows high reactivity towards cellular nucleophiles such as glutathione and the thiol groups of proteins or histones. However, 2-methylindole-3-acetic acid shows greater toxicity in combination with HRP C than IAA indicating that there could be other mechanisms of toxicity, which do not involve oxindole intermediates (Wardman, 2002). One of the cytotoxic indoles identified from in vitro screens is 6-chloroindole-3-acetic acid, a derivative with potential as a prodrug for targeted cancer therapies mediated by HRP C (Rossiter *et al.*, 2002). The challenge is to develop strategies to implement this system in vivo. The combination of HRP C and IAA derivatives offers several advantages for future antibody-gene or polymer directed enzyme prodrug therapies (Folkes and Wardman, 2001). Coupling of catharanthine and vindoline to yield α-3,4-anhydrovinblastine is a reaction catalyzed by HRP C and is of potential interest as a semisynthetic step in the production of anti cancer drugs vinblastine and vincristine from *Catharanthus roseus* (Sottomayor *et al.*, 1997). An enzyme with α-3,4-anhydrovinblastine synthase activity that shows many features characteristic of a plant peroxidase has now been purified from *C. roseus* leaves (Sottomayor *et al.*, 1998).

Ellipticine, an alkaloid isolated from *Apocyanaceae* plants and several of its more soluble derivatives exhibit promising results for clinical purposes, specially against several types of cancer which limits toxic side effects and complete lack of hematological toxicity (Stiborova *et al.*, 2001). Ellipticine is an antineoplastic agent and it has been found recently that it can covalently bind to DNA after being enzymatically activated (Frei *et al.*, 2002; Stiborova *et al.*, 2003a; 2003b). Ellipticine can be considered a drug whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Peroxidases abundant in some leukemia, i.e., myeloperoxidase (MPO) in HL-60 cells have emerged to be important candidates for ellipticine oxidation in these cells. Similarly, lactoperoxidase (LPO) that is secreted by human mammary ductal epithelial cells into

the breast duct might metabolize anti cancer drugs also. Peroxidases such as bovine LPO, human MPO and HRP oxidize the anticancer drug, ellipticine. The formation of DNA adducts from ellipticine on oxidation by peroxidases has been reported (Frei *et al.*, 2001; Poljakova and Stiborova, 2004). The importance of MPO and LPO in the oxidation of ellipticine is consistent with previous reports demonstrating that the enzymes metabolically activate a wide range of xenobiotics including drugs (Williams and Phillips, 2000).

1.4. IMMUNOAFFINITY IMMOBILIZATION

Enzyme immobilization is an important technique, which involves the retention of a biologically active catalyst within a reactor or analytical system. The immobilized enzyme retains the basic biochemical activity of the free catalyst while taking on the physical characteristics of the support. The two most important benefits of bioaffinity based enzyme immobilization are easy separation of enzyme from product and reusability of the support (Tischer and Kasche, 1999; Saleemuddin, 1999).

Immobilization very often leads to an enhancement in the resistance of the enzymes against various denaturing factors like extreme pH, temperature, high ionic strength, chemical denaturants, proteases etc. High immobilization yields, expression of high activity of the bound enzyme and stabilization against inactivation mediated by several denaturing parameters are some of the important criteria, which determine the success of any immobilization procedure. A remarkable advantage of using an antibody matrix for enzyme immobilization is the potential for replacement of enzyme inactivated during operation with a fresh preparation, enabling the recycling of the precious matrix (Solomon *et al.*, 1986).

Bioaffinity based procedures are emerging as powerful strategies for the immobilization of various enzymes (Saleemuddin and Husain, 1991; Saleemuddin, 1999; Turkova, 1999). The potential of immunnoadsorption in this regard is highly important. Specific antibodies can be raised against any enzyme in suitable experimental animals. These antibodies can be utilized after appropriate screening for the immobilization of the enzymes as antibody-enzyme insoluble complex or adsorption of the enzyme on the

antibody matrix precoupled to the support. Complexes of glucose oxidase obtained with concanavalin A and glycosyl specific antiglucose oxidase polyclonal antibodies were quite comparable in stability while complexes prepared using polyclonal antibodies raised against the native glucose oxidase were slightly less stable. The complexes of glucose oxidase obtained with glycosyl specific antiglucose oxidase polyclonal antibodies showed very high stability against inactivation mediated by the exposure of water-miscible organic solvents (Jan *et al.*, 2006). Glycosyl-specific antibodies have already proved to be very fruitful in high yield immobilization of glycoenzymes and their stabilization. Yeast invertase glycosyl-specific antibodies were raised in rabbit by immunizing them with synthesized neoglycoconjugate. These antibodies were then used for the immobilization and stabilization of invertase against several forms of denaturants (Jafri *et al.*, 1995; Jafri and Saleemuddin, 1997).

Polyclonal and monoclonal antibodies have been successfully employed for the immobilization and stabilization of enzyme on solid supports and as enzyme antibody aggregates (Jafri *et al.*, 1995). Immobilization of enzymes utilizing their adsorption on suitable immunosorbents prepared by using monoclonal and polyclonal antibodies has also been investigated (Turkova, 1999). The solid surfaces that have been utilized for immobilization of antibodies and proteins are many such as latex (Kondo and Teshima, 1995), dextran (Johnsson *et al.*, 1995), sol gel glass (Wang *et al.*, 1993), quartz (Krapivinskaya *et al.*, 1992) and carbon paste electrode (Fernandez and Costa, 1997). IgG antibodies have also been covalently bound to alginate-chitosan beads using gluteraldehyde (Albarghouthi *et al.*, 2000). Some workers have already shown the successful immobilization of enzymes on a polyclonal antibody bound support (Younus *et al.*, 2001; Khan *et al.*, 2005).

Antigens immobilized on solid supports can be used to detect or purify their corresponding antibodies from serum (Fuentes *et al.*, 2006). Direct immobilization of antigens on support surfaces (through short spacer arms) can promote interesting stabilizing effects on the immobilized antigen. HRP was immobilized on agarose by different protocols multipoint covalent immobilization involving different regions of the antigen surface. HRP immobilized preparation was much more stable than the soluble enzyme (Fuentes *et al.*, 2006). An alternative approach involves the purification of the protein (Ag) by using polyclonal antibodies bound to a support.

Although a number of procedures have been developed to immobilize antibodies on the solid matrix, some of these methods couple the antibody via chemical groups that may be important for specific recognition of an antigen. This results in a loss of functionality in a proportion of antibodies. In other methods the outcome of immobilization was coupling through unique sites in the F_c region of the antibody molecule, ensuring orientation of the antibody combining sites towards the mobile phase (Gupta *et al.*, 2003). Khan and Iqbal (2000) raised non-inhibitory antibodies against active site modified papain in rabbits and these antibodies were successfully used for the immobilization of papain on the antibodies precoupled to Sepharose. Papain IgG complex was highly stable against various forms of inactivation. Antibody immobilized on Sepharose 4B has also been widely used in the isolation and purification of target analyte in complex tissues (Lua and Chou, 2002; Zhao *et al.*, 2003).

In recent studies it has been shown that the amount of enzyme immobilized on solid supports could be raised remarkably by organizing them as alternate enzyme/antibody layers (Farooqui *et al.*, 1999). Glucose oxidase was immobilized on a glassy carbon electrode by putting alternative layers of enzyme and monoclonal antiglucose oxidase antibodies (Bourdillon *et al.*, 1994). The enzyme films that were obtained showed no substrate diffusional limitations and exhibited increased activity with layering. Large amount of glucose oxidase could be immobilized by incubating the IgG bound matrix alternatively with the enzyme and either intact IgG or $F(ab)_2$, leading to the formation of multiple enzyme layers. Immunoaffinity-layered immobilized glucose oxidase preparations, were also obtained by using polyclonal IgG and $F(ab)_2$ on Sepharose 4B and metal chelating iminodiacetate-Sepharose (Jan *et al.*, 2001).

Immunoaffinity–layered immobilized preparation was markedly more resistant to inactivation. A large assembly of glucose oxidase was made on IgG-Sepharose by alternate incubation of glucose oxidase and glycosyl specific anti-glucose oxidase polyclonal IgG. A layer-by-layer immobilization of glucose oxidase resulted in significant improvement in stability against high temperature, 4.0 M urea and high concentrations of organic solvents (Jan and Husain, 2004).

1.5. OBJECTIVES OF THE PRESENT WORK

- Peroxidases are an important group of enzymes, which have assumed importance in recent years in view of their various applications. *Momordica charantia* (bitter gourd) is an important medicinal plant available easily in most parts of India. Bitter gourd was selected for this study as an inexpensive source of peroxidase. Our objective revolved around studying some of the important characteristics of this peroxidase.
- Peroxidase from bitter gourd was purified by ammonium sulphate fractionation followed by gel filtration and affinity chromatography. A characterization of its properties such as molecular weight, stokes radius, substrate specificity, inhibitor studies, temperature and pH-optima etc were carried out.
- Since we obtained glycosylated and non-glycosylated isoenzymes, an effort was made to understand the role of glycosylation in bitter gourd peroxidase (BGP). Therefore, we have made an attempt to study the systematic comparative stability of glycosylated and non-glycosylated isoenzymes of BGP against various denaturing agents.
- In the world of peroxidases the dominance of HRP has been unquestionable. Here we have evaluated the relative stability of BGP in relation to HRP. A detailed comparative stability study of the purified BGP and commercially available HRP was also undertaken against various denaturants such as heat, pH, urea, SDS and water-miscible organic solvents. Stability of the enzymes was monitored spectrophotometrically as well as by ellipticity changes in far and near UV-circular dichroism region.
- Further, we focused on the immobilization of purified BGP on an immunoaffinity support. For this purpose the polyclonal antibodies were raised in male albino rabbits against purified BGP. The purified antibodies were coupled to CNBr-activated Sepharose 4B. Antibodies coupled Sepharose 4B was used to immobilize peroxidase from ammonium sulphate fractionated proteins of bitter gourd. Immobilized BGP was compared for its stability with soluble counterpart against heat, pH, chaotropic agents, detergents, water-miscible organic solvents and proteolytic enzyme; trypsin.

Chapter II

Purification and characterization of bitter gourd (Momordica charantia) peroxidase

2.1. INTRODUCTION

Peroxidases (E.C. 1.11.1.7) are ubiquitous heme proteins, which oxidize a variety of substrates in the presence of hydrogen peroxide. It has now been well documented that heme-containing peroxidases from plants, fungi and bacteria are evolutionary related (Welinder, 1992b). Peroxidases have diverse physiological roles in plants such as lignification, crosslinking of cell wall proteins, auxin degradation, salt tolerance and senescence. Some peroxidase genes such as those in rice are wound inducible and are involved in defense against pathogen attacks while others detoxify H_2O_2 which accumulates as a result of UV-irradiation or high light intensity (Hiraga *et al.*, 2000). Peroxidase genes and their promoters can be used for molecular breeding of useful plants (Yoshida *et al.*, 2003). Peroxidases are of great interest to researchers and are being widely used in diagnostic kits, particularly in quantification of uric acid, glucose, cholesterol and lactose (Regalado *et al.*, 2004). These enzymes are also being used in the diagnosis of diseases, for example; HRP has been used for detecting 8-dehydroxyguanosine in urine to identify bladder and prostate cancer risk (Chiou *et al.*, 2003). Such enzymes could also be employed as an efficient system for enzyme/prodrug-based anticancer approach (Greco *et al.*, 2001).

Various industrial and environmental remediation applications of peroxidases have already been described by a number of workers (Husain and Jan, 2000; Duran and Esposito, 2000). HRP is most widely studied and used among peroxidases (Dunford, 1991). Horseradish roots are expensive and are not easily available in most parts of India. Bitter gourd is easily available in most parts of the country throughout the year. It is also less expensive than horseradish.

In this study a peroxidase from bitter gourd has been purified to molecular homogeneity by using a three-step procedure; ammonium sulphate fractionation, gel filtration and affinity chromatography. Purified glycosylated BGP was characterized by investigating some hydrodynamic, kinetic, catalytic and immunological properties.

2.2. MATERIALS & METHODS

2.2.1. Materials

Bovine serum albumin, methyl α-D mannopyranoside, marker proteins, reagents for electrophoresis, cysteine, Sephacryl S-100, tropolone and ABTS were obtained from Sigma Chem. Co. (St. Louis, MO) USA. *o*-dianisidine HCl was the product of IGIB, New Delhi, India. Con A-Sepharose and Freund's adjuvants were obtained from Genei Chemicals, Bangalore India. Hydrogen peroxide was obtained from Merck, India. Ammonium sulphate, ascorbate, glucose, DTNB, guaiacol and pyragallol were purchased from SRL Chemicals, Mumbai, India. Bitter gourd was obtained from the local vegetable market. All the other chemicals and reagents used were of analytical grade and were used without any further purification.

2.2.2. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (50 g) was homogenized in a blender with 100 mL of 100 mM sodium acetate buffer, pH 5.6. The filtrate was then centrifuged at 10,000 *g* on a Remi R-24 Cooling Centrifuge. The solution thus obtained was subjected to salt fractionation by adding 50-80% (w/v) $(NH_4)_2SO_4$. The mixture was stirred overnight at 4 °C to obtain the maximum precipitate. The precipitate was collected by centrifugation at 10,000 *g* on a Remi R-24 Cooling Centrifuge. The obtained precipitate was redissolved in 100 mM sodium acetate buffer, pH 5.6 and it was dialyzed against the same buffer (Akhtar *et al.*, 2005c).

2.2.3. Purification of BGP

Sephacryl S-100 column (49x1.7 cm) was prepared. The column was equilibrated with 100 mM sodium acetate buffer, pH 5.6. In order to check uniform packing and to determine void volume of the column, 2% (w/v) solution of blue dextran prepared in 100 mM sodium acetate buffer was passed through the column. The column was thoroughly washed with 100 mM sodium acetate buffer, pH 5.6. The

dialyzed, filtered and concentrated BGP was then loaded on the column. Fractions of 2.0 mL were collected using 100 mM sodium acetate buffer, pH 5.6. The flow rate of the column was 8 mL/h. The concentration of proteins and peroxidase activity were determined in all collected fractions.

The fractions of the main peak exhibiting peroxidase activity were pooled and passed through Con A-Sepharose column. Before loading the pooled fractions, Con A-Sepharose column was equilibrated with 100 mM sodium acetate buffer, pH 5.6 containing 1.0 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.15 M NaCl. Bioaffinity adsorbed proteins were eluted by passing 100 mM sodium acetate buffer, pH 5.6 containing 0.5 M methyl α-D-mannopyranoside. The flow rate of Con A-Sepharose column was 15 mL/h. Both the columns were run at 25 °C.

2.2.4. Absorption spectra of BGP

Enzyme solution prepared in 100 mM sodium acetate buffer, pH 5.6 was scanned in the range of 240 to 460 nm. The spectrum showed two peaks of absorption maxima, one at 403 nm corresponding to heme content and the other at 280 nm for protein. R_z value was calculated by dividing the absorbance at 403 nm by the absorbance at 280 nm.

2.2.5. Effect of pH

Activity of BGP (0.4 U/mL) was determined in the buffers of different pH values. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0 and 8.0) and Tris HCl (pH 9.0 and 10.0). The percent remaining enzyme activity was calculated by taking activity at pH-optimum as control (100%).

2.2.6. Effect of temperature

Activity of BGP (0.4 U/mL) was determined at various temperatures (30-80 °C) in 100 mM sodium acetate buffer, pH 5.6. The percent remaining enzyme activity was calculated by taking activity at temperature-optimum as control (100%).

2.2.7. Assay of peroxidase activity

Peroxidase activity was estimated from the change in the optical density (λ_{460} nm) at 37 °C by measuring the initial rate of oxidation of *o*-dianisidine-HCl by hydrogen peroxide using the two substrates in saturating concentrations (Akhtar *et al.*, 2005b). Appropriate aliquots of BGP were taken in a set of test tubes. The volume was made up to 2.8 mL with 100 mM sodium acetate buffer, pH 5.6. Hydrogen peroxide (18 mM) and *o*-dianisidine (6.0 mM), 100 µL each, was added to the tubes. The total reaction volume was 3 mL in all the tubes. It was properly mixed and incubated at 37 °C for 15 min. The reaction was stopped by adding 1.0 mL 6 N HCl in each tube. The reaction volume was again mixed and the absorbance was taken at 460 nm against the reagent blank.

One unit of peroxidase activity is defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 µmol of *o*-dianisidine HCl per min at 37 °C into colored product (ε_m at 460 = 30,000 $M^{-1}cm^{-1}$).

2.2.8. Protein assay

Protein concentration was determined by using Bradford dye binding method (Bradford, 1976). Bradford dye was prepared by dissolving 30 mg of commassie brilliant blue G 250 in 15 mL ethanol and 30 mL *o*-phosphoric acid. The contents were properly dissolved in a brown bottle and the final volume was then made up to 300 mL with distilled water. Prepared dye solution was filtered through a whatman filter paper. Aliquots of protein were taken in a set of tubes and final volume was made up to 1 mL with distilled water. Bradford dye solution (5.0 mL) was then added to each tube. The color developed was read at 595 nm after 5 min incubation at room temperature against a reagent blank. Bovine serum albumin was used as a standard protein.

2.2.9. Polyacrylamide gel electrophoresis

All electrophoreses were performed by the procedure described by Laemmli (1970). Electrophoresis was carried out in vertical slab gel. Stock solution was

prepared by taking 30% acrylamide and 0.8% bisacrylamide in 1.5 M Tris-HCl buffer, pH 8.8. Stacking gels were used with a difference in the concentration of acrylamide. For stacking gels only a little more than half of the mould was filled. Concentrated stock solution of 30% (w/v) acrylamide containing 0.8% (w/v) bisacrylamide and 1.0 M Tris, pH 6.8 were mixed in appropriate proportions to the required final concentration depending on the percentage of the upper gel. Running buffer contained 1% (w/v) SDS in addition to 192 mM glycine and 25 mM Tris-HCl, pH 6.8. The gels were pre-run for 1 h before loading the samples. A constant voltage of 50 V was maintained till the tracking dye reached the bottom of the gel.

Native PAGE was run and substrate staining was performed using 18 mM H_2O_2 and 6.0 mM *o*-dianisidine-HCl. For SDS-PAGE, samples containing appropriate concentration of protein were prepared in 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol (in case of reducing conditions) and 0.001% (w/v) bromophenol blue. The samples were incubated at 100 ^{o}C for 3 min. The gel was stained for visualization by using silver nitrate solution (Oakley *et al.*, 1980). For coomassie staining, the gel was stained with five volumes of 0.25% (w/v) coomassie brilliant blue R 250 prepared in 50% (v/v) methanol and 10% (v/v) acetic acid for at least 4 h. Destaining was then performed by shaking the gels with 5% methanol and 7.5% acetic acid at room temperature.

2.2.10. Molecular weight determination

For the determination of M_r of the purified BGP, the Sephacryl S-100 column (49x1.7 cm) was calibrated with standard marker proteins of known M_r namely, BSA (66 kDa), ovalbumin (43 kDa), pepsin (35 kDa), chymotrypsin (25 kDa) and lysozyme (14.3 kDa). A graph of Ve/V_o vs Log M was plotted and the M_r of BGP was determined (Andrews, 1963). BGP was run against standard markers of known M_r on a SDS-PAGE (12.5%). Two lanes of BGP were loaded, one under reducing and the other under non-reducing conditions. The gel was silver stained for visualization of protein bands. A graph of Log M_r vs relative mobility was plotted to determine the M_r of BGP.

2.2.11. Determination of stokes radius, diffusion coefficient and sedimentation coefficient

R_S is the stokes radius of a protein. Its dimensions are g sec^{-1}. The stokes radius, R_S, is the radius of a sphere that would have the same f as the protein molecule. f is frictional coefficient, which depends on the size and shape of the protein.

$$f = 6\pi\eta R_S$$

R_S can be determined accurately by gel filtration (Siegel and Monte, 1966). Stokes radius of a protein correlates well with its elution behavior from gel filtration column. Stokes radius of BGP was determined by its elution volume from a calibrated Sephacryl S-100 column (49x1.7 cm) using 100 mM sodium acetate buffer, pH 5.6. The column was calibrated by determining the elution volume of several proteins with known stokes radii such as lysozyme (20.5 Å), chymotrypsin (22.4 Å), ovalbumin (27.3 Å) and BSA (35.6 Å). The data was analyzed according to the the equation

$$\mathbf{K_{av}} = Ve\text{-}Vo/\ Vt\text{-}Vo$$

Where, Ve = elution volume, Vo = void volume and Vt = total volume. K_{av} is the partition coefficient. The linear plot between known stokes radius and $[-\log Kav]^{1/2}$ of the marker proteins was used for the calculation of BGP's stokes radius (Laurent and Killander, 1964).

Diffusion coefficient, D is inversely proportional to f and is given by

$$\mathbf{D} = kT/f = kT/\ 6\pi\eta R_S$$

$k = 1.38 \times 10^{-16}$ erg/deg is Boltzmann's constant, T is the absolute temperature and η is the coefficient of viscosity of the medium (0.01 g/cm-sec for water and dilute aqueous salt solutions at 20 °C). D has dimensions of $cm^2\ sec^{-1}$. D provides a direct measure of frictional coefficient without any effect of mass.

The sedimentation coefficient is given by the formula

$$\mathbf{S} = M\ (1 - v_2\rho)/\ N_o\ f = M\ (1 - v_2\rho)/\ N_o 6\pi\eta R_S$$

M is the mass of the protein molecule in Da, N_o is Avogadro's number, 6.023×10^{23}. v_2 is the partial specific volume of the protein (0.73 g/cm^3), ρ is the density of the solvent (1.0 g/cm^3 for water), η is the viscosity of the solvent (0.01 g/cm-sec for water and dilute aqueous solutions at 20 °C), R_S is the stokes radius. S has the dimensions of time. S_{max} is the sedimentation coefficient for a protein having the given mass and

which is a smooth sphere having no bound water. The ratio of S_{max}/S can be used to interpret the shape of the protein (Schürmann *et al.*, 2001).

2.2.12. Substrate specificity and enzyme kinetics

Activity of BGP was determined at varying concentrations of different substrates at pH 5.6. The substrates used were *o*-dianisidine HCl (ε_m at 460 = 30,000 $M^{-1}cm^{-1}$), ABTS (ε_m at 414 = 31,100 $M^{-1}cm^{-1}$), pyragallol (ε_m at 430 = 2470 $M^{-1}cm^{-1}$), guaiacol (ε_m at 460 = 5570 $M^{-1}cm^{-1}$) and ascorbate (ε_m at 290 = 2800 $M^{-1}cm^{-1}$). V_{max} and K_m values were calculated from the Lineweaver Burk plots.

2.2.13. Carbohydrate estimation

Carbohydrate content of the purified BGP was determined using the method described by Dubois *et al.* (1956). Appropriate aliquots of BGP were pipetted into test tubes and the final volume was made to 1.0 mL by distilled water. One milliliter of 5% phenol was added in each tube. This was followed by the addition of 5.0 mL of concentrated sulfuric acid. The tubes were thoroughly mixed and allowed to stand for 20 min at room temperature. The color intensity was measured at 490 nm. Glucose was used as a standard carbohydrate.

2.2.14. Determination of total SH groups

The total SH groups in BGP were determined by the method of Sedalak and Lindsay (1968). To 0.4 mL of appropriate concentration of BGP, 2.1 mL of 100 mM Tris-HCl (pH 8.2), 0.5 mL of 10% SDS and 0.3 mL of 100 mM EDTA were added. The reaction mixture was incubated in a boiling water bath for 5 min. Then, to each tube 0.1 mL of 40 mg/100 mL DTNB prepared in methanol was added. After 30 min incubation at room temperature, the absorbance was read at 412 nm. A calibration curve with different amounts of cysteine (20-160 nmoles) was constructed by the same procedure as described above and was used to calculate the total SH groups in the samples.

2.2.15. Effect of various inhibitors

The inhibitory effect of various compounds on BGP (0.4 U/mL) was examined. The concentration of inhibitors; fluoride, EDTA, Mn^{++}, sulphide, dichromate, Co^{++}, L-cysteine and tropolone was varied from 0.01-9.0 mM. The reaction mixture was incubated for 15 min at 37 °C. The activity of the enzyme without inhibitor was considered as control (100%) for calculating percent activity. In case of azide a time dependent inactivation was monitored at concentrations of 0.1, 0.2 and 0.4 mM. The half-life obtained from this graph was plotted against inverse of azide concentrations.

2.2.16. Production of antibodies

Antibodies were raised against purified BGP in male albino rabbits. The detailed procedure has been given in Chapter V.

2.3. RESULTS

2.3.1. Purification of peroxidase from bitter gourd

Peroxidase from bitter gourd was purified to 42-fold homogeneity with a recovery of 67% of the initial activity (Table 2). A three-step scheme was employed for the purification of BGP. Bitter gourd proteins were precipitated with a high concentration of ammonium sulphate in the range of 50-80%. The precipitate was dissolved in a suitable amount of 100 mM sodium acetate buffer, pH 5.6 and was dialyzed against the assay buffer. It was then subjected to a stacking native PAGE, which was substrate stained and five isoforms of peroxidases from bitter gourd were obtained. Sephacryl S-100 column was further employed for purification; the protein–activity profile is shown in Figure 1. The pooled fractions of the peroxidase activity and protein peaks were subjected to a stacking native PAGE and three main, closely placed isoforms of the enzyme were seen after substrate staining. The bioaffinity unbound bitter gourd proteins was subjected to stacking native PAGE and revealed

Table 2: Purification steps of peroxidase from bitter gourd.

Steps	Total protein (mg)	Total activity (U)	% Yield	Specific activity (U/mg of protein)	Purification fold
Crude Homogenate	125	26125	100	209	___
$(NH_4)_2SO_4$ fractionation (50-80%)	31	21500	82	694	3
Sephacryl S-100	5	19186	73	3837	18
Con A-Sepharose	2	17538	67	8769	42

All steps for the purification of BGP were carried out at 4 °C. The crude homogenate of bitter gourd was precipitated with a high concentration of ammonium sulphate in the range of 50-80%. The dialyzed and filtered bitter gourd proteins were passed through Sephacryl S-100 column. The pooled fractions of the main sharp peak were then passed through Con A-Sepharose column. The Con A-Sepharose adsorbed glycosylated form of the enzyme was eluted from the column by passing 100 mM sodium acetate buffer, pH 5.6 containing 0.5 M methyl α-D-mannopyranoside.

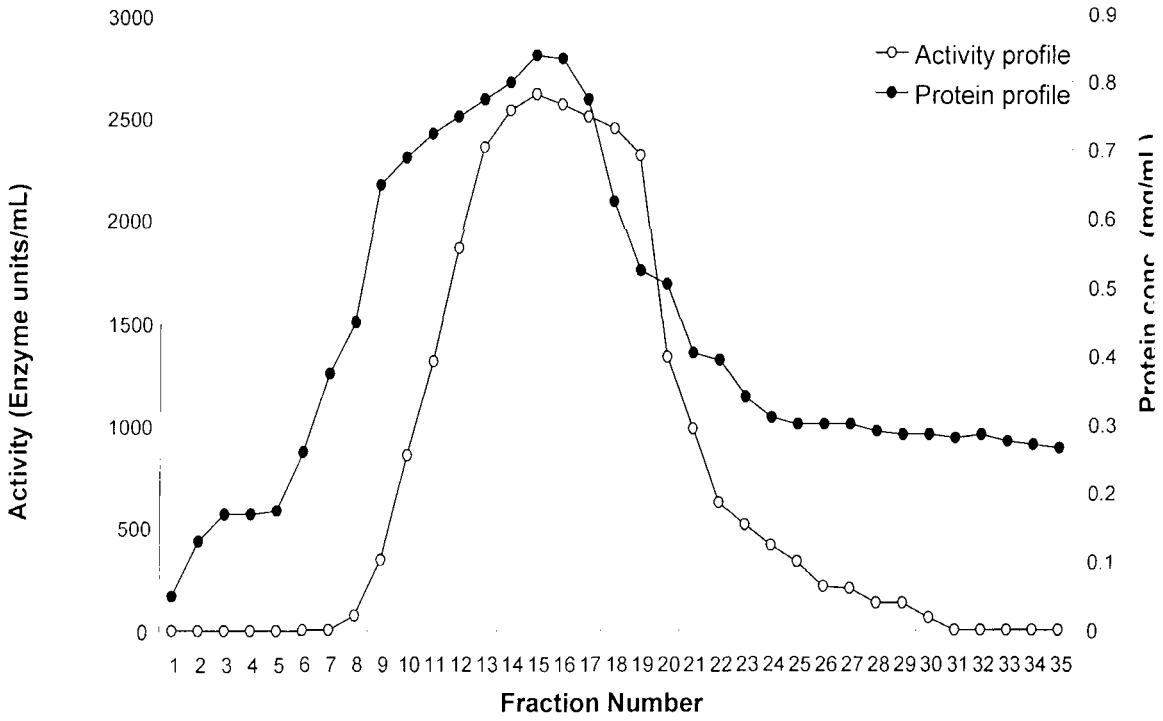


Figure 1: Elution profile of bitter gourd proteins passed through Sephacryl S-100 column

The dialyzed, filtered and concentrated bitter gourd proteins after ammonium sulphate precipitation were passed through Sephacryl S-100 column. The column was equilibrated with 100 mM sodium acetate buffer, pH 5.6. The collected fractions were assayed for peroxidase activity and protein concentration.

the presence of two closely located non-glycosylated BGP bands on substrate staining. The bound BGP on elution showed a single band on SDS-PAGE after staining by silver nitrate and when analyzed by activity staining. The analysis of step-wise purification of BGP from the homogenate to the last step on native PAGE has been demonstrated in Figure 2. A well-defined single band of BGP was obtained after silver staining on SDS-PAGE and a corresponding single band was also obtained on native PAGE when the gel was subjected to activity staining (Figure 3). The R_z (Reinheitzahl) value, the ratio of absorbances at 403 nm and 280 nm was found to be 2 (Figure 4).

2.3.2. Effect of pH and temperature on BGP activity

Purified enzyme showed temperature-optima at 40 °C (Table 3). It is a highly thermo-stable enzyme and retained nearly 50% of its initial activity even after 1 h of incubation at 60 °C. By using *o*-dianisidine HCl as substrate, BGP showed a pH-optimum at pH 5.6 (Table 3).

2.3.3. Determination of molecular weight (M_r)

The M_r of purified BGP determined by gel filtration on Sephacryl S-100 column was 43 kDa (Figure 5). The M_r was further confirmed by using SDS-PAGE under reducing and non-reducing conditions, which revealed a single band corresponding to the second band (43 kDa) of the markers (Figure 6a). The molecular weight of BGP was also calculated from the relative mobility plot of marker proteins and was found to be 43 kDa (Figure 6b).

2.3.4. Determination of stokes radius, diffusion coefficient and sedimentation coefficient of BGP

Stokes radius was determined as mentioned in the methods (Figure 7). The calculated stokes radius for BGP was 27.3 Å. The diffusion coefficient and sedimentation coefficient for BGP were calculated and found to be 8.17×10^{-7} cm^2/sec and 3.74 S (Table 3).

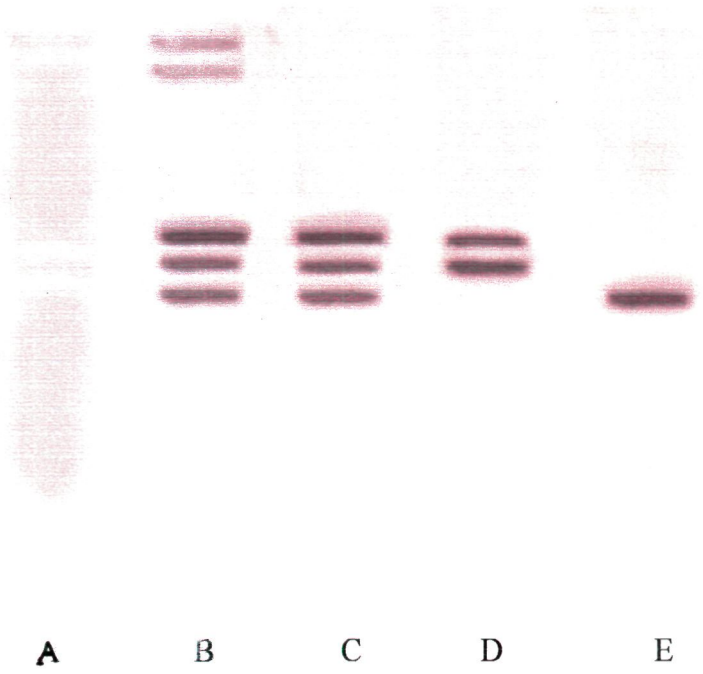
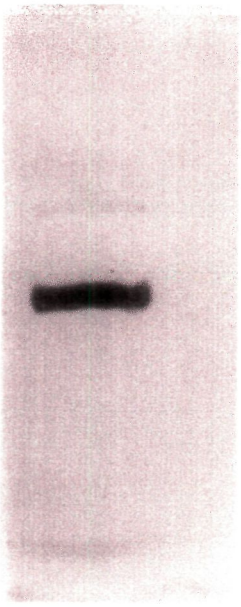


Figure 2: **BGP proteins from various purification steps**

BGP proteins were separated on 10% native PAGE. Clear homogenate (Lane A), (50-80%) ammonium sulphate fractionated proteins (Lane B), Pooled fractions of Sephacryl S-100 (Lane C), Unbound proteins from Con A-Sepharose column (Lane D), Con A-Sepharose bound and then eluted proteins (Lane E). Activity staining was performed by using 18 mM H_2O_2 and 6 mM *o*-dianisidine HCl.

(A)



(B)

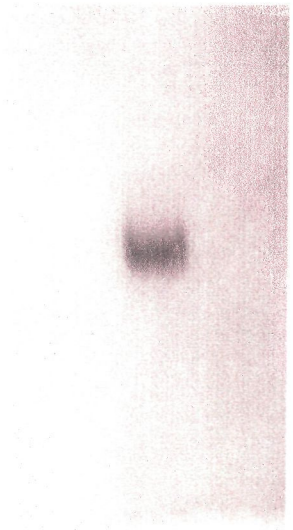


Figure 3: SDS-PAGE and activity staining of purified BGP

BGP eluted from Con A-Sepharose column was subjected to SDS-PAGE (10%) under reducing conditions subsequently; the gel was stained by silver nitrate (A). The same preparation was subjected to native PAGE (10%) and activity staining was done by using 18 mM H_2O_2 and 6 mM *o*-dianisidine HCl (B)

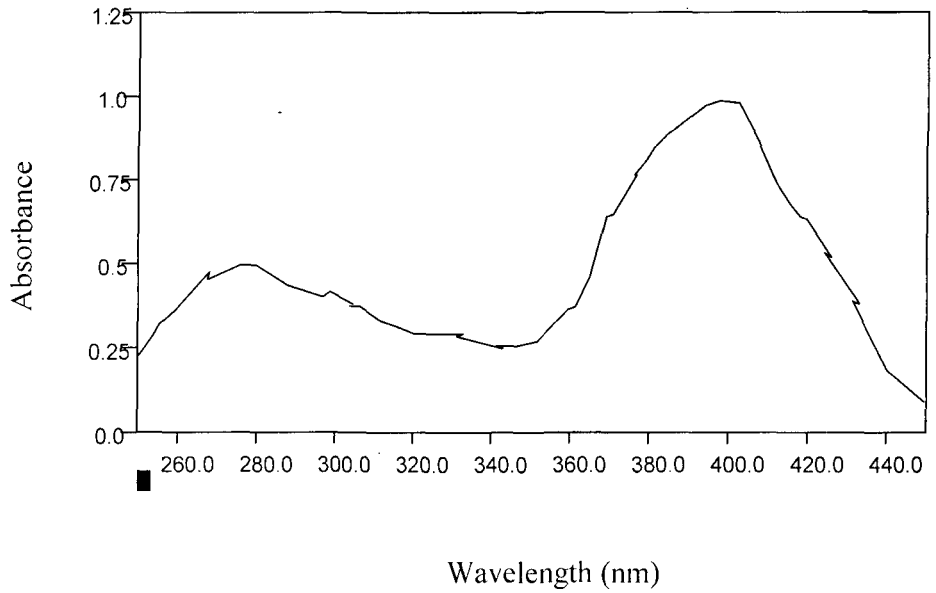


Figure 4: Absorption spectra of BGP

BGP in 100 mM sodium acetate buffer, pH 5.6 was scanned in the range of 250 to 500 nm.

Table 3: Properties of purified BGP

Properties	Result
1. Major Isoenzymes	Three
2. Glycosylated isoenzyme	One
3. Carbohydrate content	25%
4. SH groups (mmoles/mole of protein)	16
5. pH-optima	5.6
6. Temperature-optima	40 °C
7. Inhibitors	Azide, sulfide, L-cysteine
8. Molecular Weight	43 kDa
9. Stokes radius	27.3 Å
10. Diffusion coefficient	8.17×10^{-7} cm^2 /sec
11. Sedimentation coefficient	3.74 S
12. Shape	Globular

Each value represents the mean of three independent experiments performed in duplicate with average deviation not exceeding more than 5%.

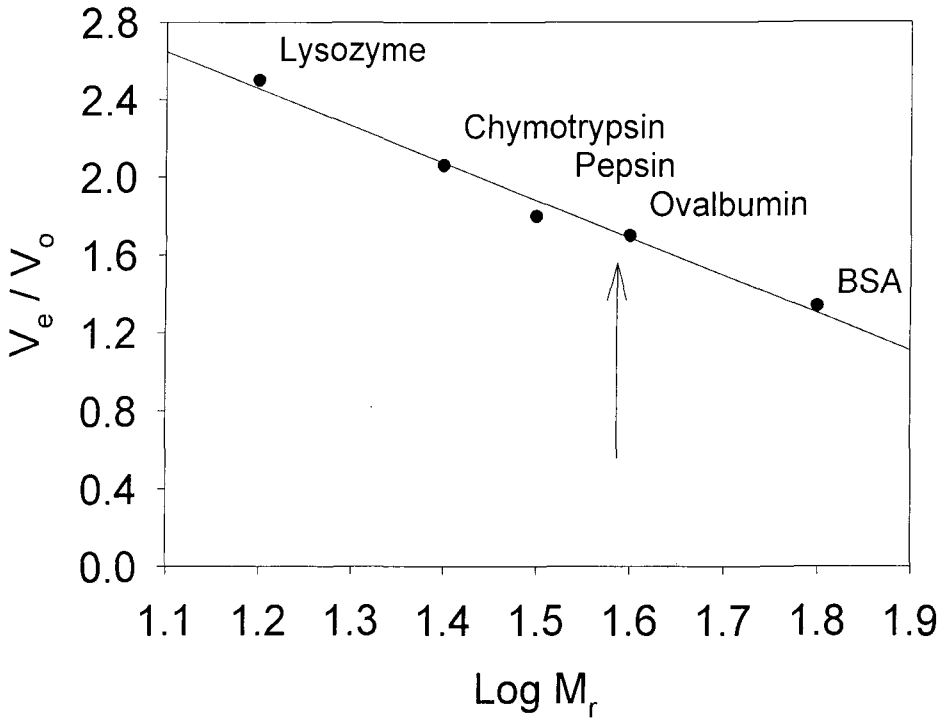


Figure 5: Determination of M_r of BGP by Sephacryl S-100 column

Sephacryl S-100 column was used to determine the elution volume of BSA, ovalbumin, pepsin, chymotrypsin and lysozyme. Molecular weights and V_e/V_o ratio of marker proteins were used for determining the M_r of purified BGP. Arrow indicates the position of the M_r of BGP.

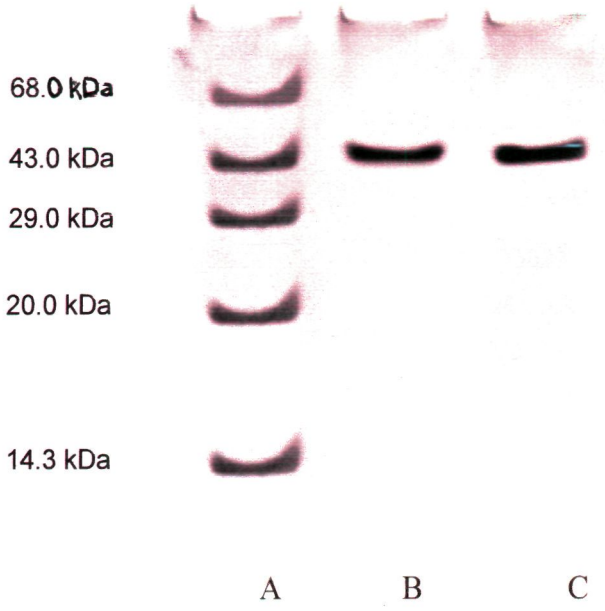


Figure 6a: Separation of purified BGP and marker proteins on SDS-PAGE

SDS-PAGE (12.5%) was used to separate purified BGP and marker proteins. The gel was silver stained after electrophoresis. Various lanes represent; Lane A: molecular weight markers, lane B: purified BGP under reducing conditions and lane C: the purified BGP under non-reducing conditions.

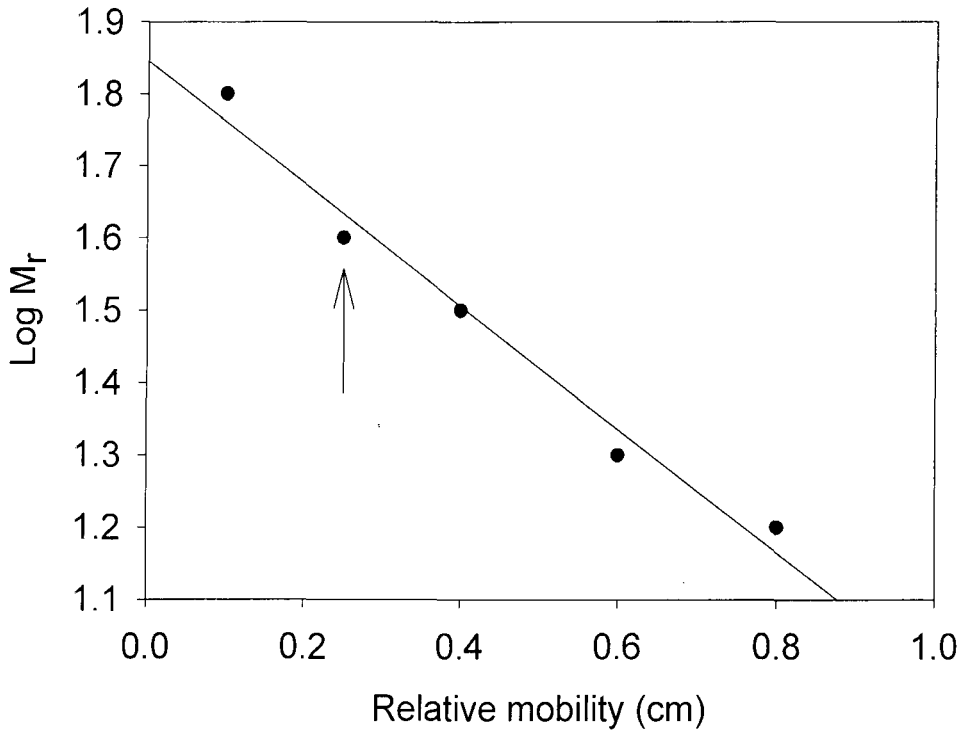


Figure 6b: Molecular weight vs relative mobility plot

The relative mobility of the standard marker proteins from the SDS gel (Figure 6a) were plotted against logarithm of M_r using least square analysis. Arrow indicates the position of the M_r of BGP.

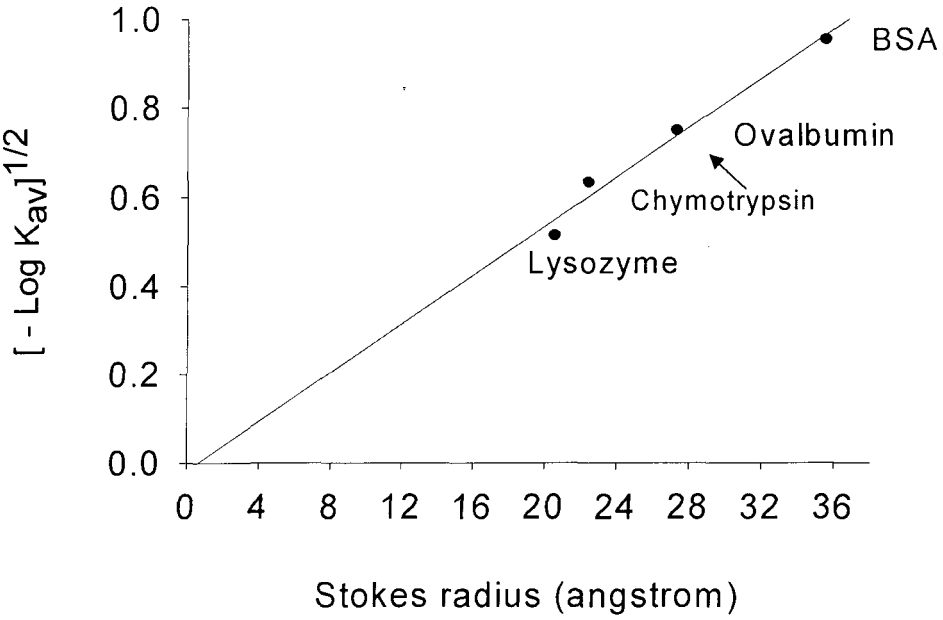


Figure 7: Determination of stokes radius of BGP

Elution volumes of marker proteins (lysozyme, chymotrypsin, ovalbumin and BSA) with known stokes radii was determined by calibrating Sephacryl S-100 column. The partition coefficient was then determined for each marker protein. Stokes radius was then determined from the plot.

2.3.5. Substrate specificity

Lineweaver-Burk plot showed an apparent K_m of 1.3 mM for *o*-dianisidine HCl. The K_m and k_{cat} values obtained for *o*-dianisidine HCl, ABTS, guaiacol and pyragallol have been given in Table 4.

2.3.6. Estimation of carbohydrate and SH group content of BGP

The carbohydrate and SH group were determined and found to be 25% (w/w) of the protein mass and 16 mmoles/mole of protein, respectively (Table 3).

2.3.7. Effect of various inhibitors

Various compounds which were reported to be inhibitors of HRP such as fluoride, EDTA, dichromate, Mn^{++} and Co^{++} were tested and were found to be non-inhibitory towards BGP (Table 5). Our studies revealed that BGP lost 67% of the original enzyme activity in the presence of 0.01 mM of azide. A complete loss of activity is evident at 0.24 mM of azide (Figure 8). BGP retained only 9.8% and 15% of the initial activity, in the presence of 0.01 mM sulfide and L-cysteine, respectively (Figure 8). Time-dependent inactivation of BGP by sodium azide was carried out at concentrations of 0.1, 0.2 and 0.4 mM. However, there was a complete loss of enzyme activity even after 15 min of incubation with 0.4 mM azide whereas a marginal activity of 10% and 2.8% was retained at 0.1 and 0.2 mM azide concentration under similar incubation conditions, respectively. There is a pseudo first order, concentration and time-dependent inactivation of the enzyme (Figure 9). A replot of the half-life ($t_{1/2}$) at sodium azide concentrations vs the reciprocal of the azide concentration yields a straight line (Figure 10). The k_{inact} from this plot was calculated to be 0.23 while the binding constant K_i, was 1.74 mM. BGP was irreversibly inhibited by azide.

Table 4: Kinetic parameters of BGP with different substrates

Substrate	Km (mM)	k_{cat} (s^{-1})
o-dianisidine	1.3	2.7
ABTS	4.9	2.0
Pyragallol	5.0	0.83
Guaiacol	5.2	2.0

Four known substrates of peroxidases; *o*-dianisidine, ABTS, pyragallol and guaiacol were taken and the velocity of the reaction at different concentrations of the substrates was used to plot the Lineweaver-Burk plots. K_m and k_{cat} were then determined from these plots. Each value represents the mean of three independent experiments performed in duplicate with the average standard deviation not exceeding more than 5%.

Table 5: Inhibitory effect of different compounds on BGP.

Inhibitors	Effect
1. Fluoride	--
2. EDTA	--
3. Dichromate	--
4. Mn^{++}	--
5. Azide	+
6. Sulfide	+
7. Co^{++}	--
8. L-cysteine	+
9. Tropolone	--

Different substances were tested for their inhibitory effect on the activity of BGP. The symbol (--) indicates that the concentration was varied from 0.01-9.0 mM. The symbol (+) indicates that the inhibitory effect was evident at lower concentrations (0.01 to 0.2 mM). The activity without inhibitor was considered as control (100%) for calculating percent activity. Each value represents the mean of three independent experiments performed in duplicate with average standard deviation not exceeding more than 5%.

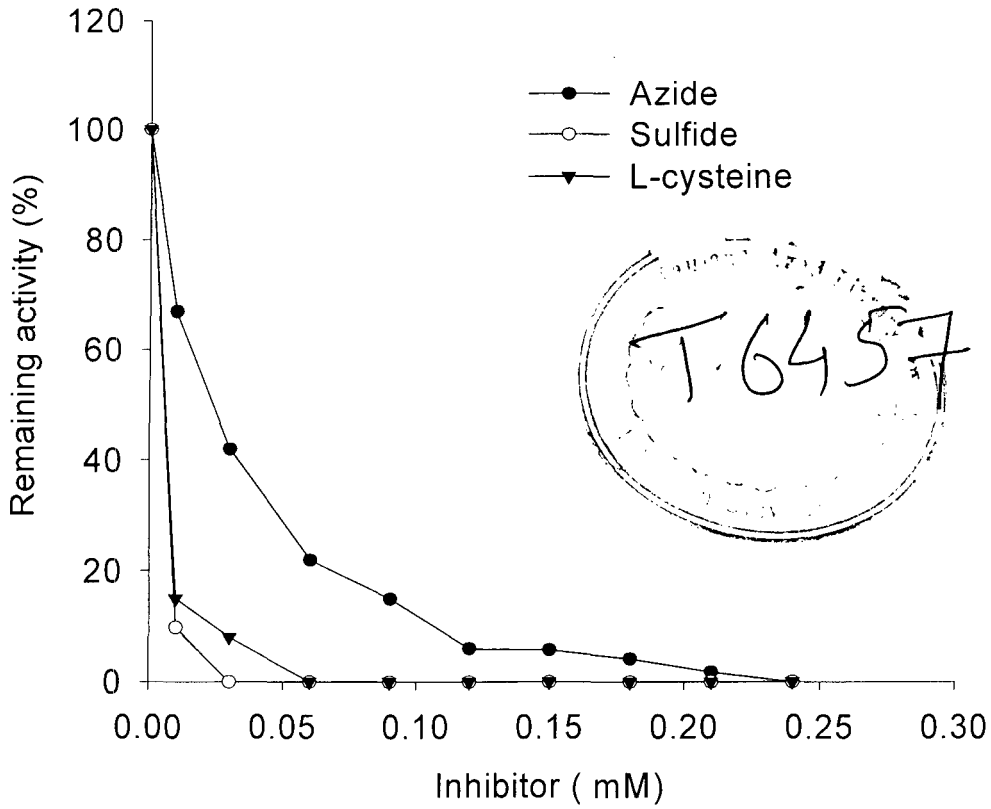


Figure 8: Effect of different inhibitors on BGP activity

Purified BGP (0.4 U/mL) was incubated at different concentrations (0.01-0.24 mM) of azide, sulfide and L-cysteine for 15 min at 37 °C. The activity without inhibitor was considered as control (100%) for calculating percent activity.

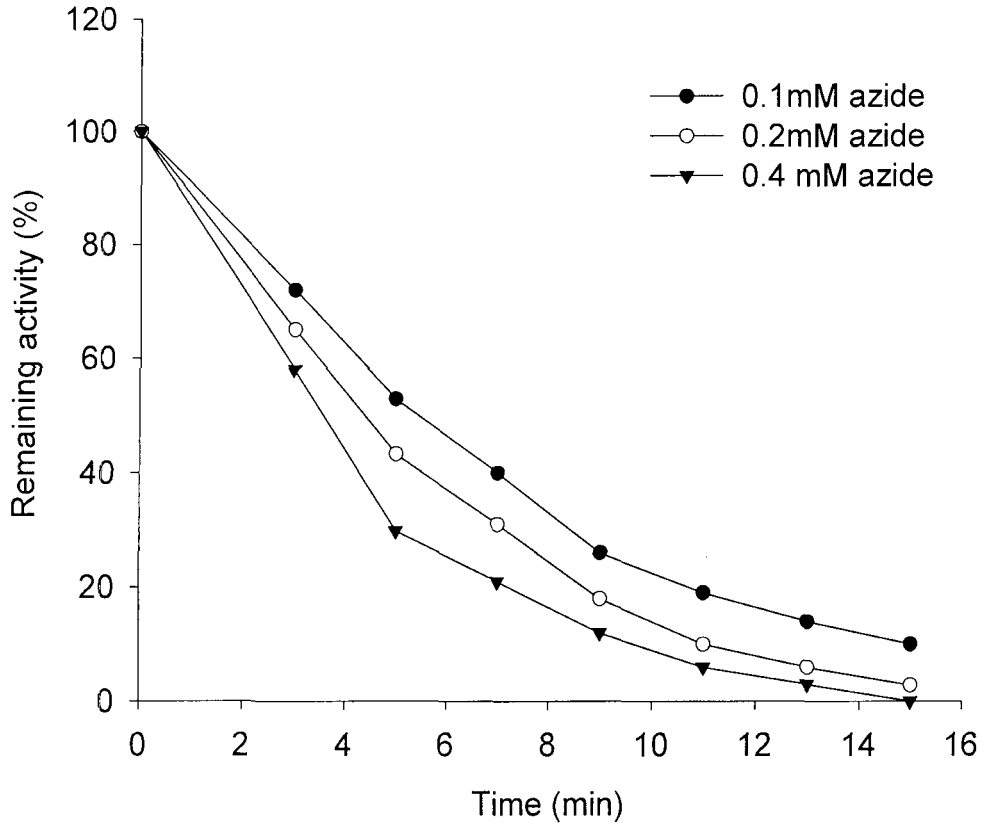


Figure 9: **Time and concentration dependent inactivation of BGP by sodium azide**

Purified BGP (0.4 U/mL) was incubated with different concentrations of sodium azide; 0.1, 0.2 and 0.4 mM, respectively. The percent activity was determined at different time intervals spanning 15 min. The activity of enzyme without inhibitor was considered as control (100%) for determining percent activity.

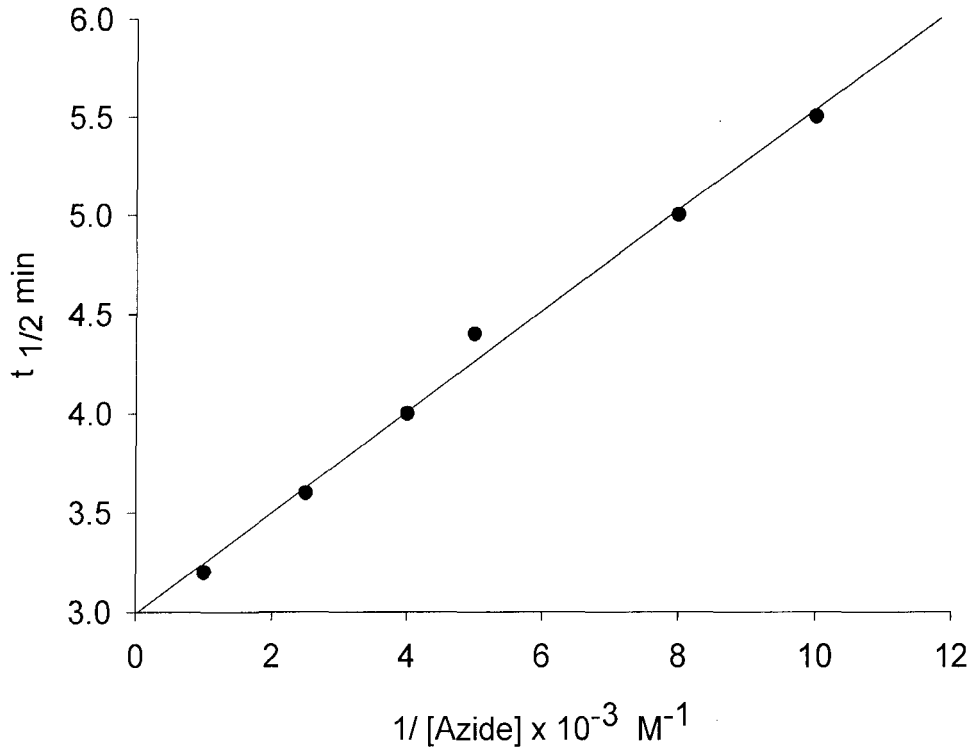


Figure 10 **Time required for inactivating half of the enzyme vs the reciprocal of the azide concentration**

The time required to inactivate 50% of BGP was calculated from Figure 10. The $t_{1/2}$ was plotted against the reciprocal of the azide concentration.

2.3.8. Production and testing of antibodies against BGP

Antibodies against purified BGP were raised in rabbit. The production of antibodies was tested by immunodiffusion in which the antiserum formed precipitin bands against BGP (Figure 11). There was a cross reaction with HRP and turnip peroxidase (data not given).

2.4. DISCUSSION

Bitter gourd is a medicinally important plant (Giron *et al.*, 1991; Lans and Brown, 1998). Reports are also available to indicate that bitter gourd is an antidiabetic, antiherpes, antipolio, anticancer and anti-HIV (Grover and Yadav, 2004). It is an inexpensive and easily available plant product in most parts of Asia. Hence, it was chosen as a source of peroxidase.

A simple three-step procedure was employed for the purification of peroxidase from bitter gourd. A modest R_z (Reinheitzahl) value of 2 was obtained. BGP shares its properties with typical class III peroxidases. The purified BGP (obtained as a single band) was found to be glycosylated. The two other isoenzymes obtained were non-glycosylated. Studies performed on BGP in our lab have revealed that it has higher stability than the already established HRP (Chapter IV). Hence, BGP has potential to replace HRP in various biotechnological applications.

The pH-optimum at pH 5.6 indicates that BGP can function in acidic environment of the plant such as vacuoles (Deepa and Arumughan, 2002). Peroxidases purified from various sources have their pH-optima in the range of 4.5-6.5. SBP has pH-optima of 5.6 (Kamal and Behere, 2003). The pH-optimum for strawberry peroxidase was 6.0 (Civello *et al.*, 1995). Acidic pH-optima have been reported for several peroxidases obtained from plant species (Nair and Showalter, 1996). The enhanced thermal stability could be due to presence of high carbohydrate residues. Many soluble peroxidases have been shown to have temperature-optima between 30 ^{o}C and 60 ^{o}C (Nair and Showalter, 1996; Bernards *et al.*, 1999). Most of the peroxidases to date are monomers. For example, rice peroxidase (48 kDa) and cotton peroxidase (48 kDa) (Deepa and Arumughan, 2002).

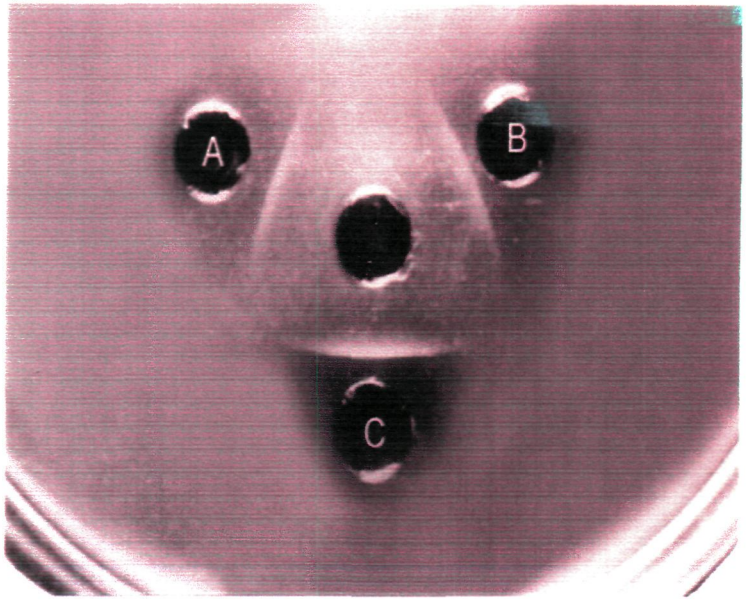


Figure 11: Immunodiffusion of BGP

Anti-BGP polyclonal antibodies were produced in male albino rabbits. Immunodiffusion was performed in 1% agarose gel prepared in normal saline. The central well contains antigen while the wells A, B and C contain equal volume of antiserum.

HRP has been reported to be a monomeric plant enzyme with a M_r of 44 kDa (Chattopadhyay and Mazumdar, 2000). Most of the class III peroxidases have molecular masses in a range of 28 to 60 kDa (Hiraga *et al.*, 2001).

Stokes radius of BGP was found to be 27.3 Å. It has earlier been reported that stokes radius of HRP was 30 Å (Fu *et al.*, 1997). The S_{max}/S ratio of BGP was 1.2. This hydrodynamic parameter indicated that BGP was a globular protein since this ratio was very close to that of ovalbumin (1.27).

The K_m value for guaiacol as obtained for oil palm leaf peroxidase was 4.0 mM (Deepa and Arumughan, 2002). The K_m with guaiacol for isoperoxidase PC3 from scented geranium callus was found to be 7.3 mM (Lee *et al.*, 2001). The apparent K_m for ABTS of the HRP isoenzyme has been reported to be 4.0 mM (Hiner *et al.*, 1996). Ascorbate did not emerge as a substrate for BGP. Class III peroxidases are known to exhibit a very poor activity for ascorbate (Kvaratskhelia *et al.*, 1997). BGP had high affinity for *o*-dianisidine HCl as compared to ABTS, pyragallol and guaiacol.

Class III peroxidases are glycosylated (Lagrimini *et al.*, 1987; Johansson *et al.*, 1992.). HRP, TP, Japanese radish peroxidase and oil palm leaf peroxidase have been reported to contain 18%, 12-18%, 20% and 37% carbohydrate, respectively (Deepa and Arumughan, 2002). A number of studies have already been performed on the inhibitory effect of azide on HRP (Ortiz de Montellano *et al.*, 1988). Sodium azide has been shown to be a potent inhibitor of many hemeprotein-catalyzed reactions (Kvaratskhelia *et al.*, 1997). HRP in the presence of sodium azide and hydrogen peroxide mediates one electron oxidation of azide ions forming azidyl free radicals, which bind covalently to the heme moiety thus inhibiting the enzyme activity (Tatarko and Bumpus, 1997). It has been earlier described that HRP was inhibited by sulfide at a concentration of 10^{-5} M (Theorell, 1951). There was no effect of Ca^{++} and Mn^{++} ions on the activity of BGP. Our results were in agreement as reported by some earlier workers that the catalytic activity of certain plant peroxidases was not influenced by calcium or manganese ions (Loukili *et al.*, 1999).

The cross reaction of BGP with other peroxidases suggested that antigenic groups on the surface of BGP were quite similar to those of HRP and TP. The glycoproteins (including peroxidases) are highly antigenic and do lead to cross reactivity (van Huystee and MacManus, 1998).

Chapter III

A role of glycosyl moieties in the stabilization of bitter gourd peroxidase

3.1. INTRODUCTION

It is now well documented that glycosylation plays an important role in the stabilization of proteins. It has become an interesting field of research for many years. The covalent attachment of carbohydrate to proteins is a very common co or post-translational event in the biosynthesis of glycoproteins (Charlwood *et al.*, 2001). The period of proteomics has followed the era of genomics bringing back the study of glycans into focus (van Huystee *et al.*, 2002). Glycosylated proteins also play an important role in mediating a number of biological processes and 'specific recognition' events (Varki, 1993). Glycosylation has been shown to be involved in protein folding, biological activity, protein stability and immunogenicity (Zeleny *et al.*, 1999). Glycosylated residues in plants could have similar functions or an extension of these roles (Zhang *et al.*, 2004). Plant glycoproteins seem to carry the same limited set of structures regardless of the species (Wilson *et al.*, 2001). Asparagine N-linked glycans are commonly found in plant glycoproteins (Repka, 2000). It has earlier been reported that plant N-glycans carry a fucose residue in α 1, 3-linkage to the innermost GlcNAc and a xylose residue is also present (Lerouge *et al.*, 1998). Even though protein glycosylation is so abundant in nature, a lot still remains to be explored in terms of how the carbohydrate attachments affect peptide and protein activity (Seitz, 2000).

A number of studies have been performed on glycosylated and deglycosylated/non-glycosylated proteins (Meier *et al.*, 1998, Rasheedi *et al.*, 2003). Among plant proteins, most peroxidases have been reported to be glycosylated and are an ideal model to further explore the significance of glycosylated residues in relation to proteins (van Huystee and Mcmanus, 1998, Hu and van Huystee, 1989). A number of peroxidases such as HRP, SBP, TP, lupin extensin peroxidase and cationic peanut peroxidase are glycosylated (Lige *et al.*, 2001; Price *et al.*, 2003). Three forms of anionic peroxidase purified from cucumber cotyledons have been shown to be glycosylated (Repka, 2000). More recently, all three isoenzymes of the peroxidase purified from broccoli stems have been found to be glycosylated (Thongsook and Barrett, 2005).

In the present study, a comparative investigation of enzymatic activity and structural stability of glycosylated and non-glycosylated isoforms of BGP was carried out over a wide range of temperature, pH, detergents and organic solvents. The stability profiles of the two forms of BGP exposed to high urea concentrations was also studied in detail by employing fluorescence.

3.2. MATERIALS & METHODS

3.2.1. Materials

Sephacryl S-100, methyl α-D mannopyranoside and reagents for electrophoresis were obtained from Sigma Chem. Co, (St. Louis, MO) USA. *o*-dianisidine-HCl was the product of IGIB, New Delhi, India. Hydrogen peroxide was obtained from Merck India. Ammonium sulphate, urea, dimethyl sulphoxide, dimethyl formamide and sodium dodecyl sulphate, Triton X-100, Tween-20 were purchased from SRL Chemicals, Mumbai, India. Con A-Sepharose was obtained from Genei Chemicals, Bangalore India. Bitter gourd was obtained from the local market. All the other chemicals and reagents used were of analytical grade.

3.2.2. Preparation of glycosylated and non-glycosylated BGP

Bitter gourd proteins were precipitated by ammonium sulphate (Akhtar *et al.*, 2005b). Further purification of BGP was carried out by employing gel filtration on Sephacryl S-100 column (49x1.7cm) and affinity chromatography on Con A-Sepharose (Chapter II). The unbound BGP obtained in the washing buffer represented the non-glycosylated preparation. Bioaffinity adsorbed proteins were eluted by passing 100 mM sodium acetate buffer, pH 5.6 containing 0.5 M methyl α-D-mannopyranoside. The eluted protein was called glycosylated BGP.

3.2.3. Measurement of peroxidase activity

Peroxidase activity was estimated by the procedure described in Chapter II.

3.2.4. Protein assay

The Bradford method was used for the determination of protein concentration as described in Chapter II.

3.2.5. Polyacrylamide gel electrophoresis

All electrophoreses were performed as described in Chapter II. A SDS-PAGE (10%) was also run and stained for glycoprotein by using the Periodic Acid-Schiff (PAS) reagent (Zacharius *et al.*, 1969).

3.2.6. Effect of temperature

Activity of glycosylated and non-glycosylated preparation of BGP (0.4 U/mL) was determined at various temperatures (30-80 °C) in 100 mM sodium acetate buffer, pH 5.6. The percent remaining enzyme activity was calculated by taking activity at temperature-optimum as control (100%).

In another set of experiment, glycosylated and non-glycosylated BGP preparations were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.6 for varying times. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and then the peroxidase activity was determined as described in text. The percent remaining activity was determined by considering 60 °C unincubated enzyme as control (100%).

3.27. Effect of pH

An appropriate and equal amount of glycosylated and non-glycosylated BGP preparations were added for determining the activity of enzyme in the buffers of different pH values. The buffers used were glycine-HCl (2.0 and 3.0); sodium acetate buffer (4.0 -6.0); sodium phosphate (7.0, and 8.0) and Tris HCl (9.0 and 10.0). The remaining percent activity was calculated by taking activity at optimum-pH as control (100%).

3.2.8. Effect of urea

Glycosylated and non-glycosylated BGP (0.4 U/mL) were incubated with increasing concentrations of urea (2.0-8.0 M) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 2 h.

In another set of experiment, both preparations of BGP (0.4 U/mL) were incubated with 4.0 M urea for varying time intervals. Peroxidase activity was determined after each incubation period. The activity of the urea untreated enzyme was considered as control (100%) for calculating the remaining percent activity.

3.2.9. Effect of organic solvents

Glycosylated and non-glycosylated BGP (0.4 U/mL) were incubated with varying concentrations of water-miscible organic solvents, DMSO or DMF (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of the enzyme without organic solvent was taken as control (100%) for the calculation of remaining percent activity.

3.2.10. Effect of detergents

Glycosylated and non-glycosylated BGP (0.4 U/mL) were incubated with varying concentrations of detergents; SDS (0.1-1.0%, w/v), Tween-20 (0.5-5.0%, v/v), Triton X-100 (0.5-5.0 %, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of the enzyme without detergent was considered as control (100%) for the calculation of percent remaining activity.

3.2.11. Fluorescence measurements

Fluorescence measurements were performed on a Shimadzu Spectrofluorometer, Model RF-540. Sample solutions containing 0.2 mg/mL concentration of BGP were incubated with varying concentrations of urea (0-8.0 M) at 30 °C for 6 h.

The intrinsic fluorescence was taken by exciting the protein (0.1 mg/mL) at 280 nm and the fluorescence emission spectra was recorded between 300-400 nm for each denaturant concentration.

The average emission wavelength, λ av.em was calculated according to the equation

$$\lambda_{av.em} = \Sigma (Fi\ \lambda i) / \Sigma Fi$$

where Fi is the fluorescence intensity and λ is the wavelength, this parameter reflects changes in the shape of the spectrum as well as in position (Royer *et al.*, 1993).

3.3. RESULTS

3.3.1. Glycosylated and non-glycosylated forms of BGP

Sephacryl S-100 column was employed for the purification of ammonium sulphate fractionated bitter gourd proteins, the pooled fractions of the activity and protein peaks were subjected to a stacking native PAGE and three, closely placed isoforms of BGP were obtained on substrate staining (Figure 2, Chapter II).

The unbound BGP obtained from the Con A-Sepharose column exhibited the presence of two non-glycosylated bands of BGP on substrate staining (Chapter II, Figure 2). The bound glycosylated proteins eluted from the support showed a single band when stained on native gel (Chapter II, Figure 2). The eluted protein also gave a single band on SDS-PAGE when stained with silver nitrate. Glycosylated BGP showed a pink colored band when gel was stained with PAS reagent (Figure 12).

3.3.2. Temperature-activity profiles

Both forms of BGP showed same temperature-optima at 40 °C. However, glycosylated BGP retained significantly more activity than non-glycosylated preparation at temperatures other than temperature-optima. Glycosylated BGP showed greater fraction of catalytic activity, 80% at 60 °C while the non-glycosylated BGP retained only 65% of the initial activity. Moreover, glycosylated BGP exhibited 74% activity at 70 °C whereas non-glycosylated preparation lost nearly 50% activity at the same temperature (Figure 13).

3.3.3. Thermal denaturation

Glycosylated BGP was significantly more stable than non-glycosylated preparation when incubated for different time intervals at 60 °C. The former retained 42% activity after 1 h of incubation at 60 °C whereas the latter exhibited marginal activity of 14% under similar incubation conditions (Figure 14).

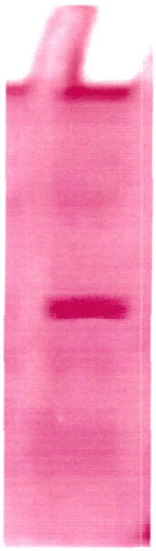


Figure 12: PAS staining of glycosylated BGP

Purified BGP was separated on 10% SDS PAGE. The peroxidase was stained by using the procedure described in the methods.

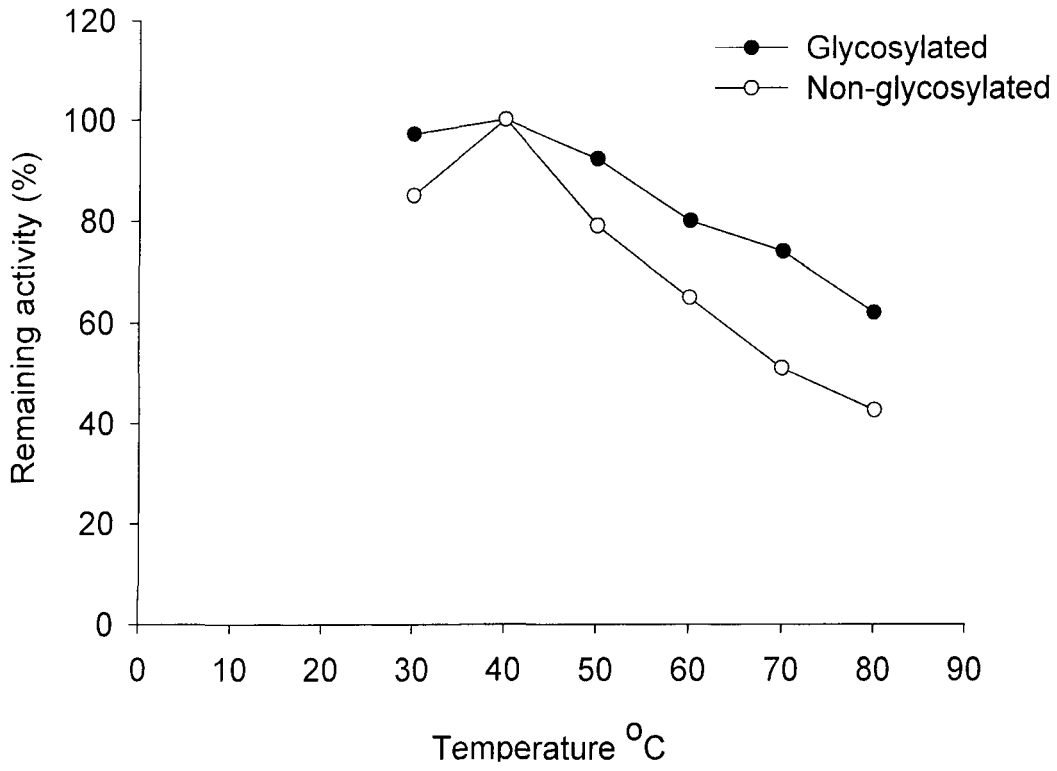


Figure 13: Temperature-activity profiles for glycosylated and non glycosylated BGP

Glycosylated and non-glycosylated preparations of BGP (0.4 U/mL) were assayed at various temperatures (30-80 °C) in 100 mM sodium acetate buffer, pH 5.6. Activity expressed at 40 °C was taken as control (100%) for calculating percent activity.

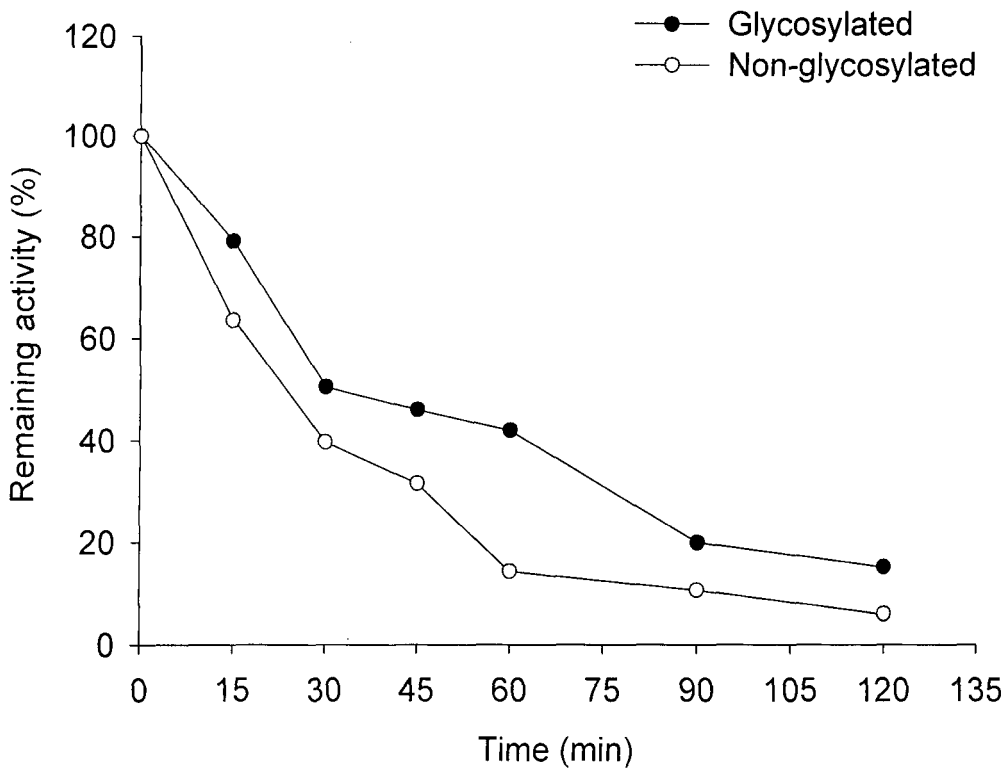


Figure 14: Thermal denaturation of glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated preparations of BGP (0.4 U/mL) were incubated at 60 °C for varying times in 100 mM sodium acetate buffer, pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined as described in the text. Un-incubated samples at 60 °C were taken as control (100%) for the calculation of percent activity.

3.3.4. Effect of pH

The pH-activity profiles of glycosylated and non-glycosylated BGP showed no alteration. Both enzyme preparations exhibited similar pH-optima (Figure 15). The far-UV-CD spectra of both forms at different pH values were also identical (data not given).

3.3.5. Effect of urea

The effect of different concentrations of urea on both forms of BGP has been demonstrated in Figure 16. There was no significant change in the activity of both types of BGP after their incubation with 2.0 M urea for 2 h. However, the change in catalytic activity became more pronounced from 4.0 M urea concentration onwards. Glycosylated form retained a remarkably high activity, 90% after exposure to 6.0 M urea for 2 h whereas non-glycosylated forms lost nearly 68% of the original activity under similar incubation conditions. Further exposure of both types of BGP with 8.0 M urea for 2 h resulted in a significant loss of 71% activity by non-glycosylated preparation whereas glycosylated BGP retained 88% of the initial activity under similar exposure.

Figure 17 shows the urea-induced inactivation of glycosylated and non-glycosylated BGP. Glycosylated BGP was remarkably more resistant to inactivation induced by 4.0 M urea as compared to non-glycosylated BGP. Glycosylated BGP retained 90% activity even after 2 h incubation with 4.0 M urea while the non-glycosylated preparation showed 40% of the initial activity under identical treatment.

3.3.6. Effect of organic solvents

The effect of increasing concentrations of water-miscible organic solvents; DMSO and DMF, (10-60%, v/v) on the activity of glycosylated and non-glycosylated BGP is shown in Table 6. There was a conspicuous activation in both glycosylated and non-glycosylated preparations of BGP when treated with water-miscible organic

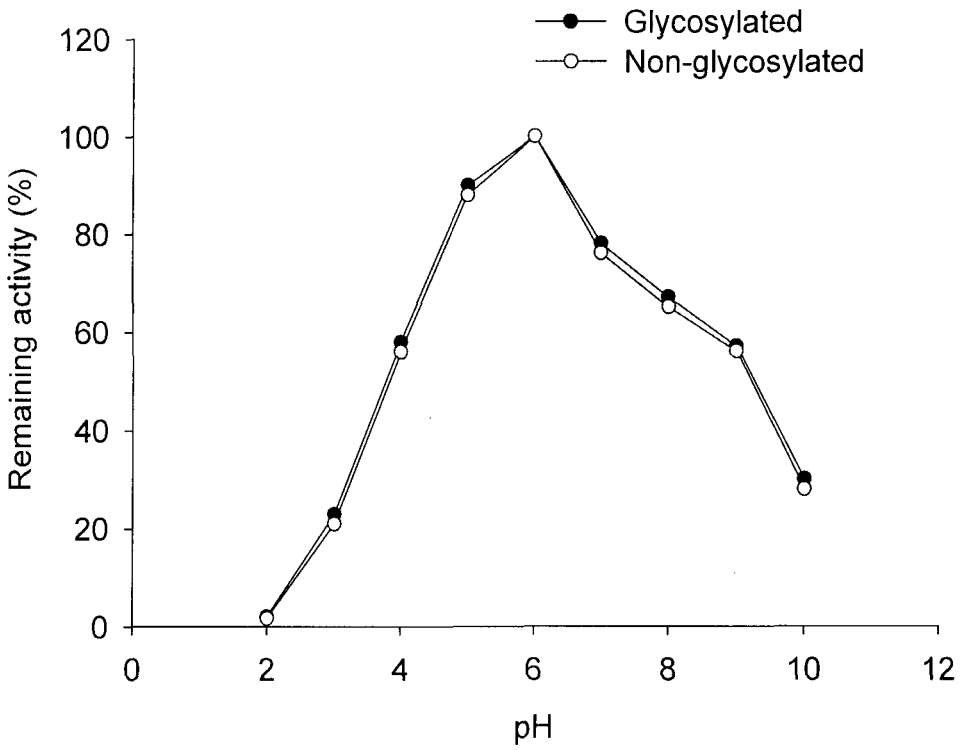


Figure 15: pH-activity profiles for glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated preparations of BGP (0.4 U/mL) were assayed in the buffers of different pH values. The buffers used were glycine HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0 and 8.0) and Tris-HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Activity expressed at pH 6.0 was taken as control (100%) for calculating percent remaining activity.

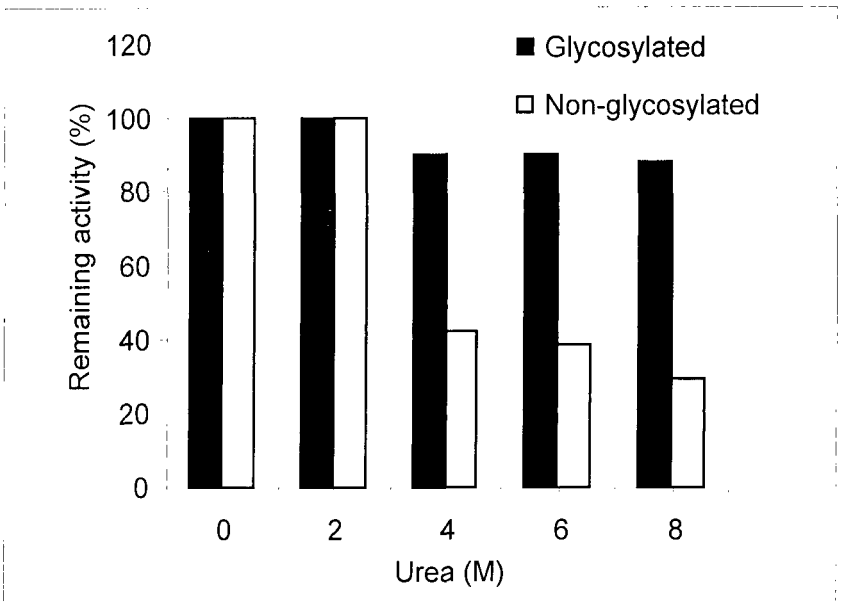


Figure 16: Effect of urea concentration on glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated preparations of BGP (0.4 U/mL) were incubated in 2.0-8.0 M urea in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 2 h. Enzyme activity was determined as mentioned in the text. For calculating the remaining percent activity urea untreated samples were considered as control (100%).

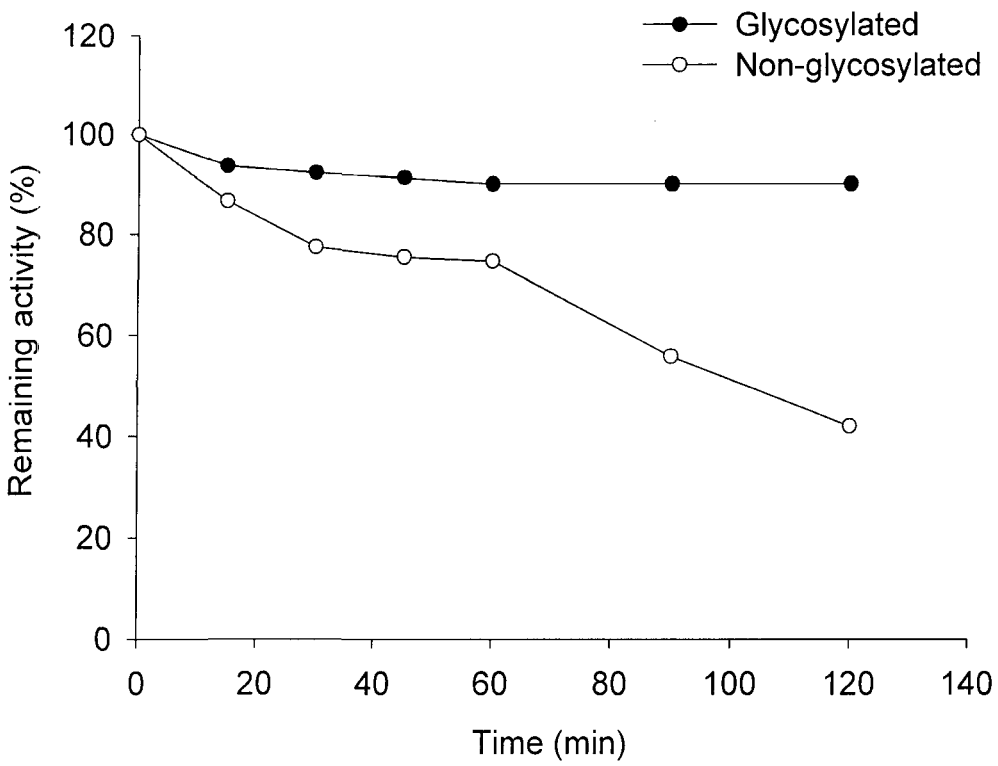


Figure 17: Effect of 4.0 M urea on glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated preparations of BGP (0.4 U/mL) were incubated in 4.0 M urea in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for varying times. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the remaining percent activity urea untreated samples were considered as control (100%).

Table 6: Effect of DMF and DMSO on glycosylated and non-glycosylated BGP.

Organic solvent (%, v/v)	Percent remaining activity			
	DMF		DMSO	
	Glycosylated	Non-glycosylated	Glycosylated	Non-glycosylated
0	100	100	100	100
10	92	85	121	109
20	130	126	203	149
30	137	127	209	132
40	142	112	174	125
50	142	106	146	113
60	142	106	110	96

Glycosylated and non-glycosylated BGP (0.4 U/mL) were incubated with increasing concentration of DMSO and DMF (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation not exceeding more than 5%.

solvents. The activity of glycosylated BGP enhanced to 209% as compared to non-glycosylated preparation which exhibited an enhancement up to only 132% when treated with 30% (v/v) DMSO. Glycosylated BGP activity was increased to 110% in the presence of 60% (v/v) DMSO whereas non-glycosylated preparation showed no activation. Exposure to 40% (v/v) DMF resulted in an enhancement of glycosylated BGP activity to 142% while the non-glycosylated BGP exhibited a slight enhancement in enzyme activity. The activity of glycosylated and non-glycosylated BGP was enhanced to 142% and 106%, respectively at exposure to 60% (v/v) DMF.

3.3.7. Effect of detergents

The effect of increasing concentrations of SDS (0.1-1.0%, w/v) on the activity of both types of BGP has been shown in Figure 18. The activity of glycosylated BGP was enhanced significantly to 231% by the exposure of 1.0% (w/v) SDS whereas the non-glycosylated preparation was activated to 112% in the presence of 0.4% (w/v) SDS. The activity of glycosylated form was enhanced markedly to a very high value, 786% at 0.3% (w/v) SDS whereas the non-glycosylated preparation was activated to less than half of this value at the same concentration of SDS.

Pre-incubation of both types of BGP with Tween-20 (0.5-5.0%, v/v) at 37 °C for 1 h resulted in a greater loss of activity for non-glycosylated form than the glycosylated form (Figure 19). Glycosylated BGP preparation retained more than 50% activity at a concentration of 2.5% (v/v) Tween-20 whereas the non-glycosylated preparation lost nearly 62% activity under similar exposure. Glycosylated BGP showed 44% of the catalytic activity at a concentration of 5.0% (v/v) Tween-20 while the non-glycosylated form retained a marginal activity of 22% under similar incubation conditions.

The effect of increasing concentration of Triton X-100 (0.5-5.0%, v/v) is depicted in Figure 20. The peroxidase activity of glycosylated BGP was enhanced till a concentration of 1.5% (v/v) and reached to 146% and 143% at 0.5% (v/v) and 1.0% (v/v) Triton X-100 respectively. The non-glycosylated BGP preparation was activated to 117% by 0.5% (v/v) Triton X-100. Glycosylated form retained a higher fraction of enzyme activity as compared to the non-glycosylated form, for example it retained

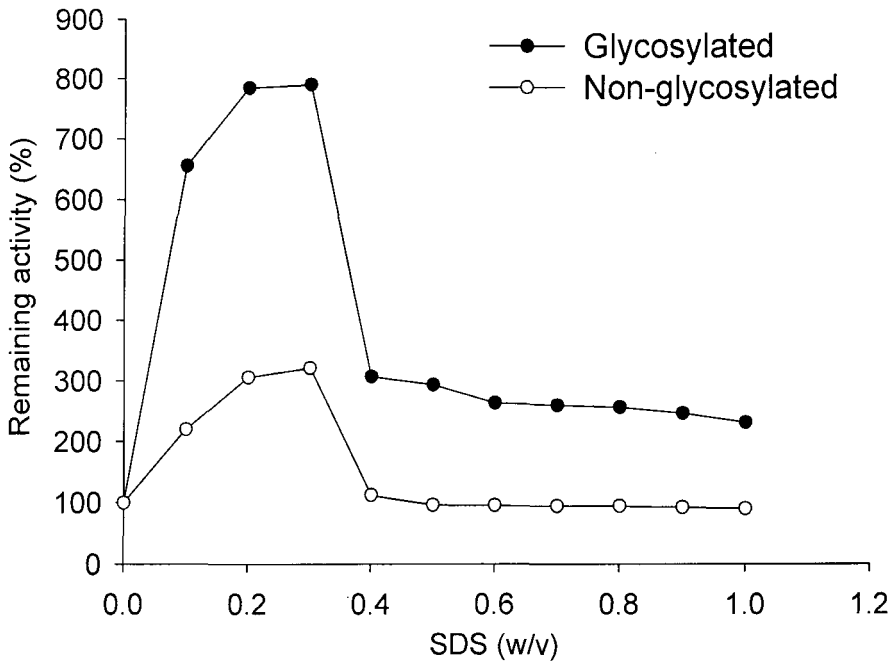


Figure 18: Effect of SDS on glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated preparations of BGP (0.4 U/mL) were incubated with increasing concentration of SDS (0.1-1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. The activity of SDS unexposed enzyme preparations was taken as control (100%) for the calculation of percent remaining activity.

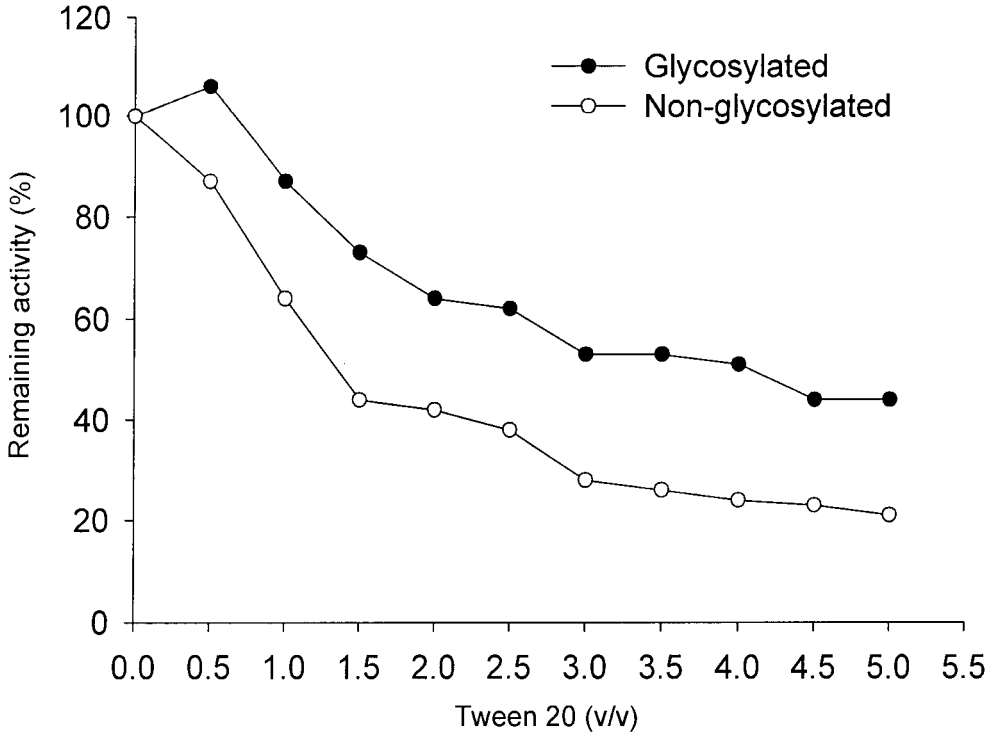


Figure 19: Effect of Tween-20 on glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated BGP preparations (0.4 U/mL) were incubated with increasing concentration of Tween-20 (0.5-5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. The activity of Tween-20 untreated enzyme preparations was taken as control (100%) for the calculation of percent remaining activity.

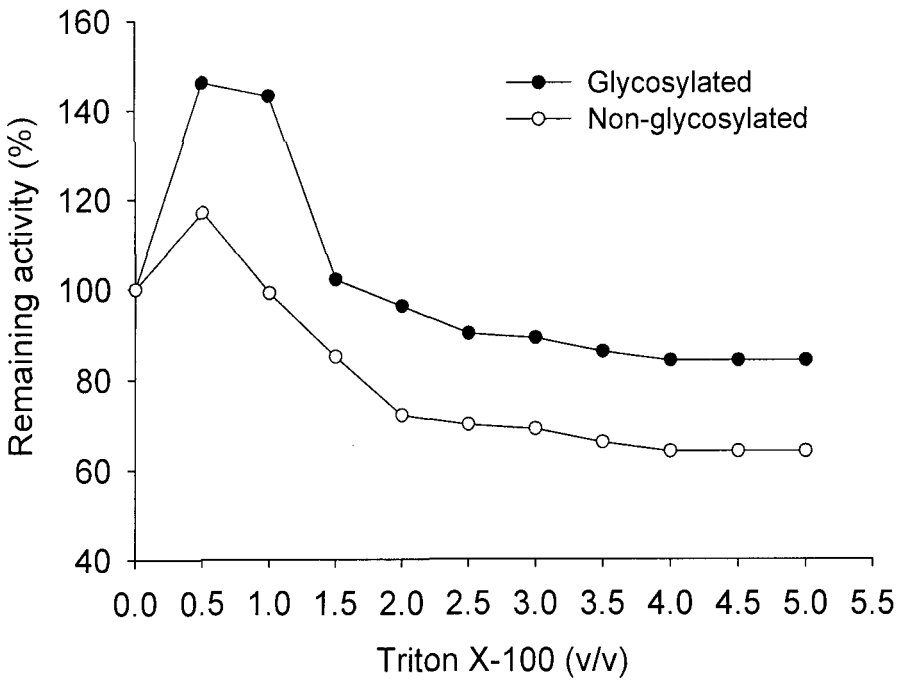


Figure 20: Effect of Triton X-100 on glycosylated and non-glycosylated BGP

Glycosylated and non-glycosylated BGP preparations (0.4 U/mL) were incubated with increasing concentration of Triton X-100 (0.5-5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. The activity of Triton X-100 untreated enzyme preparations were taken as control (100%) for the calculation of percent remaining activity.

84% activity at 5.0% (v/v) Triton X-100 whereas the latter had only 63% activity under identical incubation conditions. There was a leveling off in the activity of both glycosylated and non-glycosylated preparation of BGP from 3% (v/v) Triton X-100. The intrinsic fluorescence of glycosylated and non-glycosylated preparations of BGP has been shown in Figure 21.

3.3.8. Fluorescence studies of urea treated BGP

Figure 22 demonstrates the unfolding pathway of glycosylated and non-glycosylated BGP in the presence of high concentrations of urea (0-8.0 M). There was an increase in fluorescence intensity (Fi) for both forms of BGP when treated with 1.0-2.0 M urea as compared to Fi of control. The maximum increase in Fi was at 1.0 M urea. The non-glycosylated preparation showed a higher Fi (205 a.u) at 1.0 M urea as compared to the glycosylated form (136 a.u). The glycosylated form exhibited a leveling off of Fi from 5.0-7.0 M urea, which was followed by a decline in the Fi at 8.0 M urea. This constant phase was absent in case of non-glycosylated preparation, which showed a decline in Fi from 136 a.u at 5.0 M urea to 99 a.u at 6.0 M urea. The glycosylated and non-glycosylated form had an almost identical Fi of 20 a.u and 24 a.u, respectively at 8.0 M urea. At each urea concentration (0-7.0 M) the non-glycosylated preparation exhibited a higher Fi as compared to glycosylated form.

The fluorescence λ av.em has been plotted and analyzed as a function of high urea concentration (Figure 23). There was only a slight change (1-2 nm) in λ av.em as compared to control in case of both glycosylated and non-glycosylated BGP from 1.0-3.0 M urea. However, in both forms of BGP the change was more pronounced from 4.0 M to 8.0 M urea (3-12 nm). The λ av.em was slightly higher for non-glycosylated preparation than glycosylated form at each urea concentration.

The ratio of Fi at 306 nm and 350 nm ($F_{306/350}$) using an excitation wavelength of 280 nm can be regarded as contribution of tyrosine residues to fluorescence measurements (Sanchez del Pino and Fersht, 1997). A decrease in the $F_{306/350}$ ratio was an indication of a red shift while an increase in the ratio was a signal of blue shift in the environment of tyrosine residues.

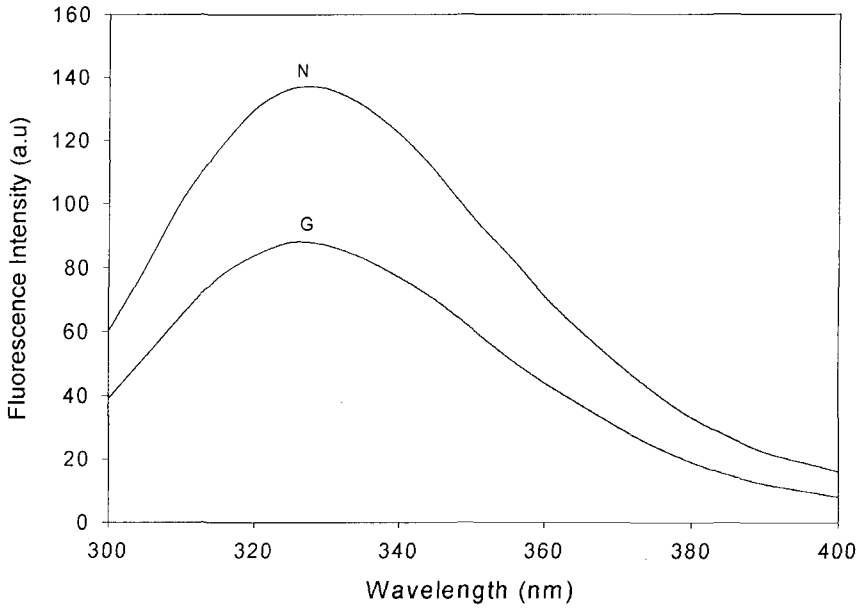


Figure 21: Intrinsic fluorescence of glycosylated and non-glycosylated BGP

The solutions of glycosylated and non-glycosylated BGP preparations were prepared in 100 mM sodium acetate buffer, pH 5.6 and their intrinsic fluorescence was performed on Shimadzu Spectrofluorometer, model RF-1501. The protein concentration of 0.1 mg/mL was chosen. The excitation wavelength was 280 nm and the emission wavelength was taken between 300-400 nm. The G represents glycosylated BGP and the N represents non-glycosylated BGP preparation.

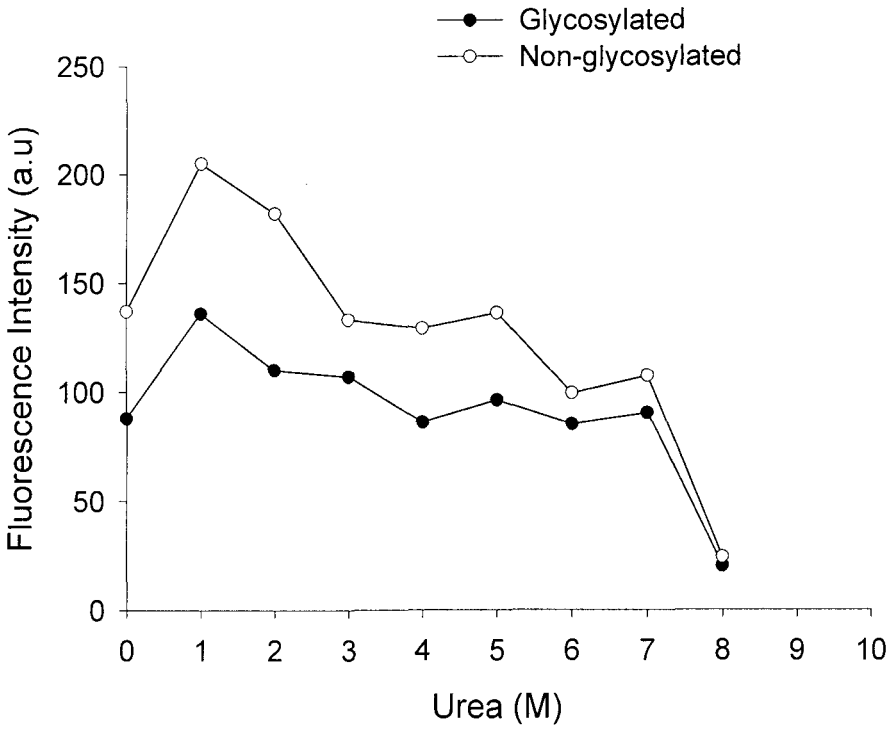


Figure 22: Effect of high urea concentration on fluorescence intensity of glycosylated and non-glycosylated BGP

The fluorescence intensity (a.u) at high urea concentration (0-8.0 M) was measured for glycosylated and non-glycosylated BGP. The protein concentration was chosen to optimize the measuring conditions. The protein was excited at 280 nm. The preparations without urea were taken as control.

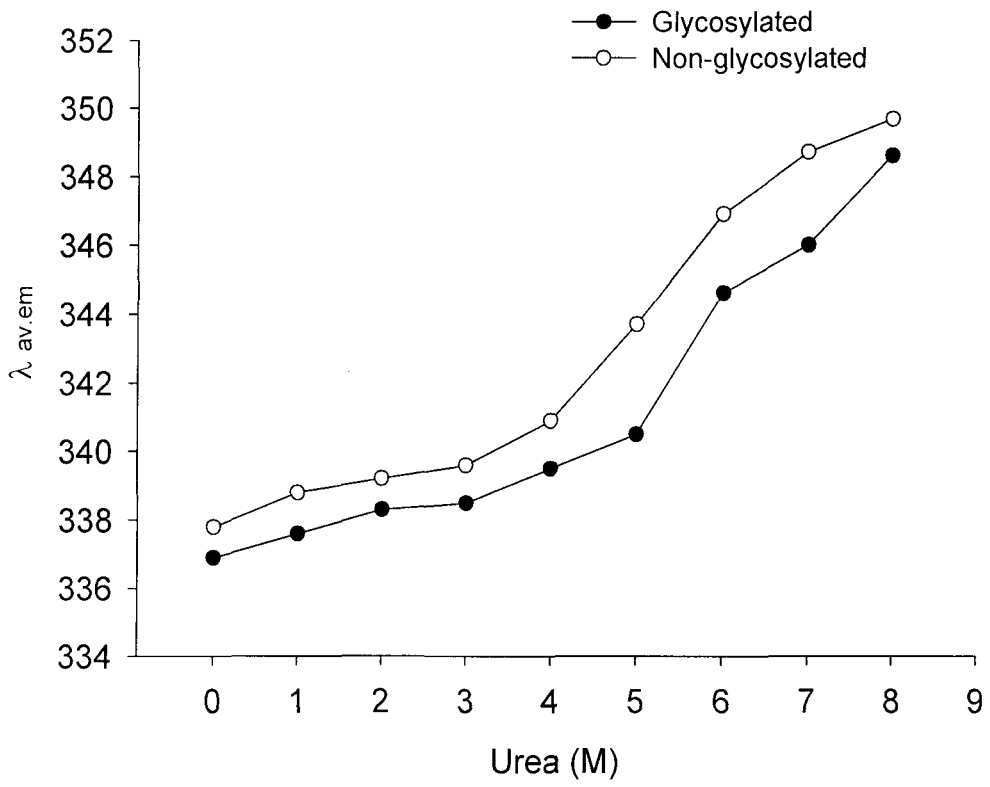


Figure 23: The average wavelength emission (λ av.em) for glycosylated and non- glycosylated BGP at high urea concentration

The average wavelength emission (λ av.em) of 0-8.0 M urea treated glycosylated and non-glycosylated BGP preparations were measured. The protein concentration was chosen to optimize the measuring conditions. The proteins were excited at 280 nm. The preparations without urea were taken as control.

A plot between $F_{306/350}$ and urea concentration (0-8.0 M) has been demonstrated in Figure 24. The $F_{306/350}$ was 0.85 from 1.0-3.0 M urea in glycosylated BGP while this ratio was significantly decreased from 0.83 at 1.0 M urea to 0.71 at 4.0 M urea in case of non-glycosylated preparation. At 5.0 M urea the $F_{306/350}$ ratio in the case of non-glycosylated BGP was 0.55 whereas it was 0.76 for glycosylated form. Thus the decrease in $F_{306/350}$ ratio when compared to control was more for non-glycosylated BGP as compared to glycosylated BGP. Further, the ratio at 8.0 M urea was nearly same as that for glycosylated form (0.3).

3.4. DISCUSSION

Glycosylation is one of the most naturally occurring modifications of the covalent structure of proteins (Jafari-Aghdam *et al.*, 2005). The effect of glycosylation on the activity of enzymes has been described by various workers (Rudd *et al.*, 1994). Several roles have been suggested for the carbohydrate moiety of proteins one of which includes stabilization of protein conformation (Wang *et al.*, 1996). The present study evaluates the role of carbohydrate moieties in the stability of BGP. Glycosylated BGP was more stable than the non-glycosylated preparation over a wide range of temperatures (Figure 13). Both forms of BGP exhibited the same temperature-optima. The form devoid of carbohydrate residues has more flexibility and therefore, it showed diminished stability at higher temperatures (Jafari-Aghdam *et al.*, 2005). The absence of glycans has a profound influence on thermo-stability of proteins (Pathirana *et al.*, 2005). It has also been reported by earlier workers that glycosylation of various enzymes resulted in their thermal stabilization (Gu *et al.*, 1989; Takegawa *et al.*, 1998). The thermal denaturation of both forms of BGP at 60 ^{o}C for various time intervals showed greater retention of enzyme activity for glycosylated BGP (Figure 14). Carbohydrate moieties thus clearly indicate a significant role in the stabilization of enzymes against thermal denaturation (Mer *et al.*, 1996). There was not much difference in the pH-activity profiles of glycosylated and non-glycosylated BGP (Figure 15). Both types of BGP exhibited similar pH-optima.

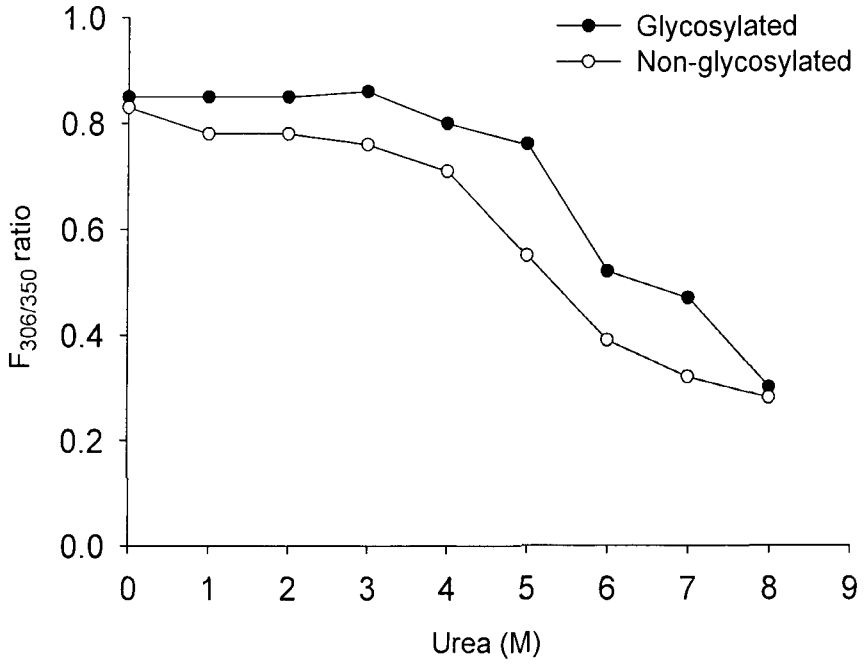


Figure 24: The ($F_{306/350}$) ratio of glycosylated and non-glycosylated BGP at high urea concentration

The ($F_{306/350}$) ratio of 0-8.0 M urea treated glycosylated and non-glycosylated BGP preparations were measured. The protein concentration was chosen to optimize the measuring conditions. The proteins were excited at 280 nm. The preparations without urea were taken as control.

There was no detectable difference in the far UV-CD spectra of the two forms at various pH values indicating that there was no significant change in the secondary structures of the enzymes (data not shown). Glycosylated BGP retained greater enzyme activity as compared to the non-glycosylated form when treated with different concentrations of urea and when exposed to 4 M urea at different time intervals (Figures 16 and 17). Kwon and Yu (1996) have shown that the presence of carbohydrate moiety in enzymes increased resistance to inactivation caused by urea. The percent remaining activity for glycosylated form was more as compared to the non-glycosylated preparation when exposed to various concentrations of detergents; SDS, Tween-20 and Triton X-100 (Figures 18-20) and various organic solvents; DMSO and DMF (Table 6). There was a three-fold increase in the activity of SBP when the enzyme was treated with 0.1% (w/v) of SDS and Tween-20 (Flock *et al.*, 1999).

It was found that the non-glycosylated BGP exhibited higher Fi due to greater exposure of fluorophore in this form (Figure 21). Earlier workers have reported a similar increase in Fi of the enzyme devoid of carbohydrate moiety (Jafari-Aghdam *et al.*, 2005).

A close to native like form was evident at 4.0 and 6.0 M urea for glycosylated BGP and at 3.0 to 4.0 M urea in case of non-glycosylated BGP (Figure 22). At higher urea concentration Fi changed and the ($F_{306/350}$) ratio decreased (Figure 24) with a change in the λ av.em for both the forms (Figure 23). This suggested a red shift with the tyrosine residues getting exposed to a more polar microenvironment. However, the change in Fi and ($F_{306/350}$) ratio was more pronounced in case of non-glycosylated preparation indicating that possibly the glycosylated form was shielded due to steric hindrance of carbohydrate residues. At 8.0 M urea both the forms were in a similar microenvironment demonstrated by an almost similar ($F_{306/350}$) ratio. Thus there were overall changes in tertiary structure of both forms and a reorientation of tyrosine residues, which was more in the case of non-glycosylated form. This study pointed towards an important role assumed by carbohydrate moieties in stabilizing the glycosylated form of BGP.

Chapter IV

A comparative study of bitter gourd peroxidase with horseradish peroxidase

4.1. INTODUCTION

Plant peroxidases are receiving increasing attention due to their extensive bioactivation property and potential applications in clinical, biochemical, biotechnological, and related areas (Azevedo *et al.*, 2003). Advances have recently been made in using them to synthesize, under mild and controlled conditions, chiral organic molecules, which are highly valuable compounds (Colonna *et al.*, 1999). They have also been successfully employed in the development of new bioanalytical tests, improved biosensors and in polymer synthesis (Sakharov, 2004).

Peroxidases have been used for various analytical applications in diagnostic kits, such as quantification of uric acid, glucose, cholesterol and lactose. Due to its ability to convert colorless substrates into chromogenic products, these enzymes are one of the best suited for the preparation of enzyme-conjugated antibodies, which are used in enzyme-linked immunosorbent assay (ELISA) tests (Krell, 1991; Schutz *et al.*, 1997).

HRP has enormous diagnostic, biosensing and biotechnological applications (Regalado *et al.*, 2004). The availability and cost of commercially available HRP restricts its applications. Peroxidases from other plant sources have also been explored; however these investigations have been unsuccessful in terms of identifying peroxidases able to knock out HRP as the preferred plant peroxidase in biotechnology. The availability of highly stable and active peroxidases from sources other than horseradish would go a long way towards the development of a catalytic enzyme with broad commercial and environmental applications.

In this study, a systematic effort has been made to compare the stability of a homogeneously purified glycosylated peroxidase from bitter gourd and commercially available HRP. A comparative stability study was carried out at different temperatures and in buffers of varying pH values, by measuring the circular dichroism in near/far UV-CD region and the activity of the enzymes. The stability of BGP against urea, SDS and water-miscible organic solvents was also compared with HRP by monitoring the activity of the enzymes.

4.2. MATERIALS & METHODS

4.2.1. Materials

Horseradish peroxidase (205 U/mg) and bovine serum albumin were obtained from Sigma Chem. Co. (St. Louis, MO) USA. *o*-dianisidine HCl was the product of IGIB, New Delhi, India. Hydrogen peroxide was obtained from Merck, India. Ammonium sulphate, urea, dimethyl sulphoxide, dimethyl formamide and sodium dodecyl sulphate were purchased from SRL Chemicals, Mumbai, India. Bitter gourd was obtained from the local market. All the other chemicals and reagents used were of analytical grade and were used without any further purification.

4.2.2. Effect of Temperature

Activity of BGP and HRP (0.4 U/mL) was determined at various temperatures (30-80 °C) in 100 mM sodium acetate buffer, pH 5.6. The percent remaining enzyme activity was calculated by taking activity at temperature-optimum as control (100%).

In another set of experiment, BGP and HRP (0.4 U/mL) were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.6. After each incubation period, the enzyme was taken out and was chilled quickly in crushed ice for 5 min. The enzyme was brought to room temperature and then the peroxidase activity was determined at 37 °C.

4.2.3. Effect of pH

Activity of BGP and HRP (0.4 U/mL) was determined in the buffers of different pH values. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0 and 8.0) and Tris HCl (pH 9.0 and 10.0). The percent remaining activity was calculated by taking enzyme activity at pH-optimum as control (100%).

4.2.4. Effect of urea

BGP and HRP (0.4 U/mL) were incubated with increasing concentrations of urea (2.0-8.0 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 °C.

In another set of experiment, BGP and HRP preparations (0.4 U/mL) were incubated with 4.0 M urea for varying time intervals. Peroxidase activity was determined after each incubation period. The activity of the urea untreated enzyme was considered as control (100%) for calculating percent activity.

4.2.5. Effect of SDS

BGP and HRP (0.4 U/mL) were incubated with increasing concentrations of SDS (0.1-1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was determined after the incubation period. The activity of the SDS untreated enzyme was considered as control (100%) for calculating percent activity.

4.2.6. Effect of water-miscible organic solvents

BGP and HRP (0.4 U/mL) were incubated with varying concentrations of water- miscible organic solvents, DMSO and DMF (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of the enzyme without water miscible organic solvents was considered as control (100%) for calculating percent activity.

4.2.7. Assay of peroxidase activity

Peroxidase activity was estimated as described in Chapter II.

4.2.8. Protein assay

Protein concentration was determined by using the Bradford method (Chapter II).

4.2.9. CD spectroscopy

Circular dichroism measurements were made on a JASCO J-720 spectropolarimeter calibrated with D-10-camphorsulfonic acid. The instrument was equipped with a water-jacketed, thermostatically controlled cell holder, Neslab RTE circulating water bath and a microcomputer. The path length of the cell and protein concentration was chosen to optimize the measuring conditions. Equal protein concentration of BGP and HRP was taken. The samples were prepared in 100 mM sodium acetate buffer, pH 5.6. The far UV-CD spectra were taken in a wavelength region of 200-250 nm. For pH measurements the samples were prepared in glycine HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0 and 8.0) and Tris-HCl (pH 9.0 and 10.0) and the readings were taken at 25 °C. The pH of the solution was measured before every experiment.

4.3. RESULTS

4.3.1. Thermal stability

The temperature-activity profiles of BGP and HRP are depicted in Figure 25. Temperature-activity profiles of BGP and HRP exhibited similar temperature-optima. However, temperature-activity profile of BGP showed more broadening as compared to HRP. BGP retained nearly 81% of the initial activity at 60 °C whereas HRP retained only 39% activity under identical conditions.

Temperature-dependent near-CD studies of both BGP and HRP elucidated changes in tertiary structure as shown in Figure 26. There was no alteration in tertiary structure of BGP till 60 °C. However, the loss of tertiary structure in the case of HRP at all the observed temperatures was greater than BGP. The changes in the secondary structures of BGP and HRP were monitored by single wavelength analysis at 222 nm (Figure 27). There was no alteration in the secondary structure of BGP from 40 °C-70 °C. However, the loss of secondary structure of HRP was remarkably very high in comparison to BGP. HRP lost substantial secondary structure at 50 °C, which is followed by a further loss of secondary structure from 60 °C to 80 °C. BGP thus

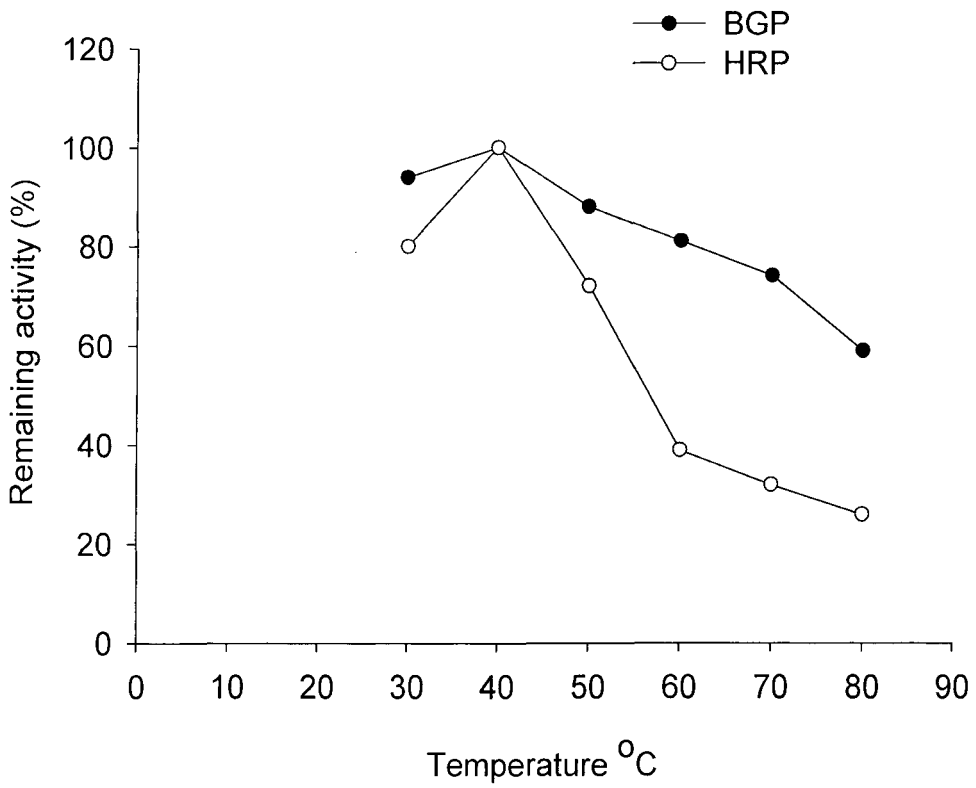


Figure 25: Temperature-activity profiles for BGP and HRP

BGP and HRP (0.4 U/mL) were assayed at various temperatures (30-80 °C) in 100 mM sodium acetate buffer, pH 5.6. Activity obtained at 40 °C was considered as control (100%) for calculating percent activity.

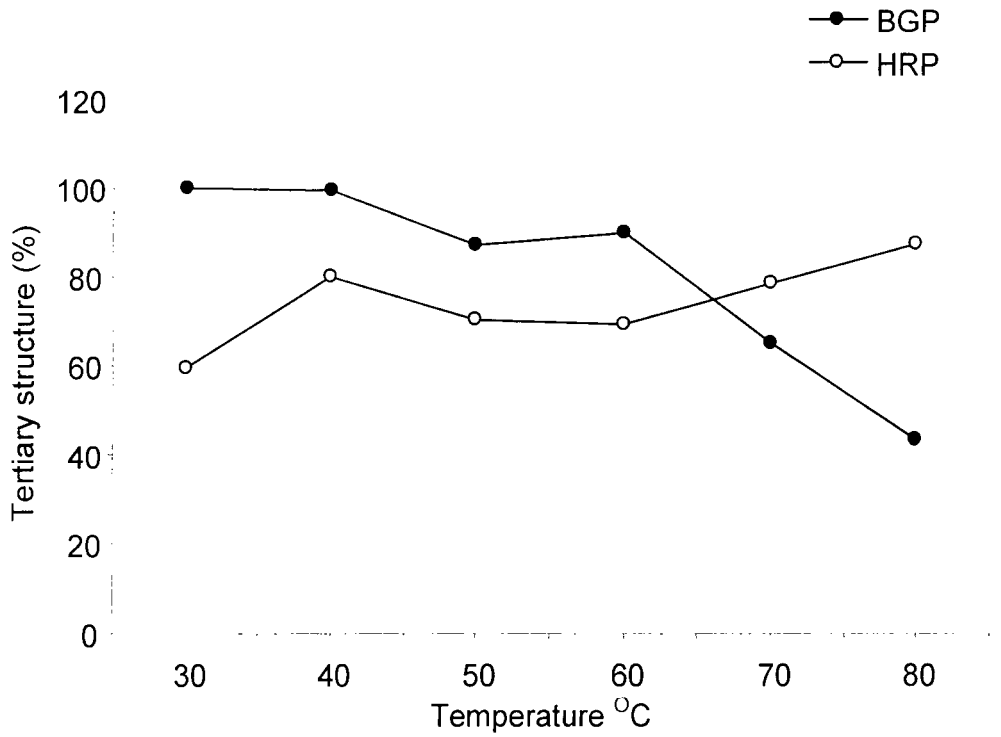


Figure 26: Comparison of tertiary structure of BGP and HRP

The percent change in tertiary structure of BGP at 263 nm and of HRP at 255 nm is plotted at various temperatures (30-80 °C). CD experiments were carried out in 100 mM sodium acetate buffer, pH 5.6. The protein concentration and path length of the cell were chosen to optimize the measuring conditions.

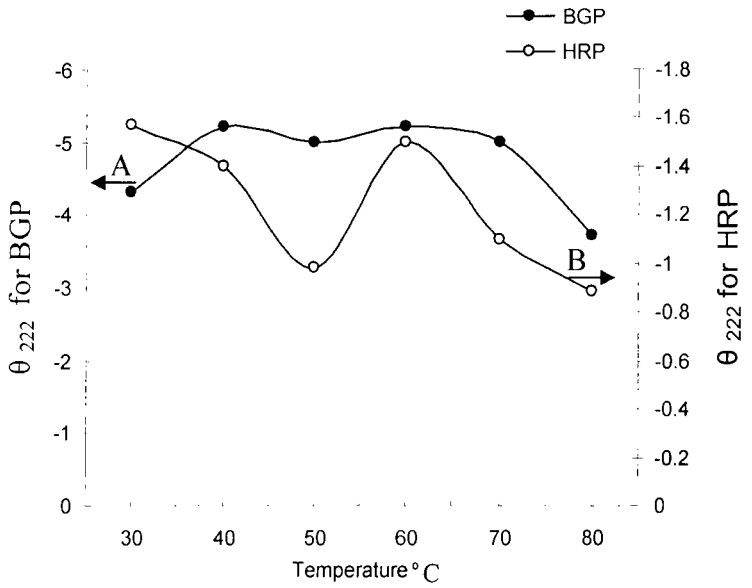


Figure 27: Comparison of secondary structure of BGP and HRP

(A) Change in the ellipticity of BGP at 222 nm with temperature.

(B) Change in the ellipticity of HRP at 222 nm with temperature.

The CD experiments were carried out in a temperature range of (30-80 ^{o}C) and in 100 mM sodium acetate buffer, pH 5.6. The protein concentration and path length of the cell were chosen to optimize the measuring conditions.

showed greater thermo-stability with respect to loss in secondary structure as compared to HRP. BGP incubated at 60 °C retained nearly 50% of the initial activity after 1 h of incubation while HRP exhibited marginally 9% activity under similar experimental conditions (Figure 28). The far UV-CD spectra of BGP at 80 °C (curve 1), 60 °C (curve 2) and 30 °C (curve 3) are depicted in Figure 29. There was a greater loss of helicity at 80 °C as compared to 30 °C and 60 °C.

4.3.2. pH stability

The pH-activity profiles of BGP and HRP are demonstrated in Figure 30. The stability of BGP was much higher in the alkaline range in comparison to HRP. In fact, HRP lost 92% of its original activity at pH 9.0 while BGP retained more than half of its activity at the same pH. A sharp decline in the stability of BGP and HRP occurred as the pH was lowered below 5.0.

The pH dependence of the enzyme activity (measured at 460 nm) and ellipticity at 222 nm of BGP are shown in Figure 31. There was an increase in the negative ellipticity from pH 2.0 onwards which reached a maximum at around pH 6.0. Further increase in pH led to the continuous decrease in the CD signal value, which reached a conspicuous minimum at pH 10.0. BGP lost all secondary contacts at pH 10.0 and assumed a completely unfolded structure. A similar pattern was observed when the BGP activity was measured at different pH values. The far UV-CD spectra of BGP at pH 6.0 and pH 10.0 have been overlaid for comparison of changes in the spectral features (Figure 32). There was a loss of secondary structure as shown by decrease in negative value of the spectrum at pH 10.0 (Figure 32, curve A). BGP retained more secondary structure at pH 6.0 than at pH 10.0 as manifested by a more negative signal and hence a higher helicity at pH 6.0.

4.3.3. Effect of chaotropic agent, urea

The inactivation of BGP and HRP at different concentrations of urea has been demonstrated in Figure 33. There was no significant change in the activity of BGP and HRP after their incubation with 2.0 M urea for 2 h. However, the change in catalytic activity became more pronounced from 4.0 M urea concentration onwards.

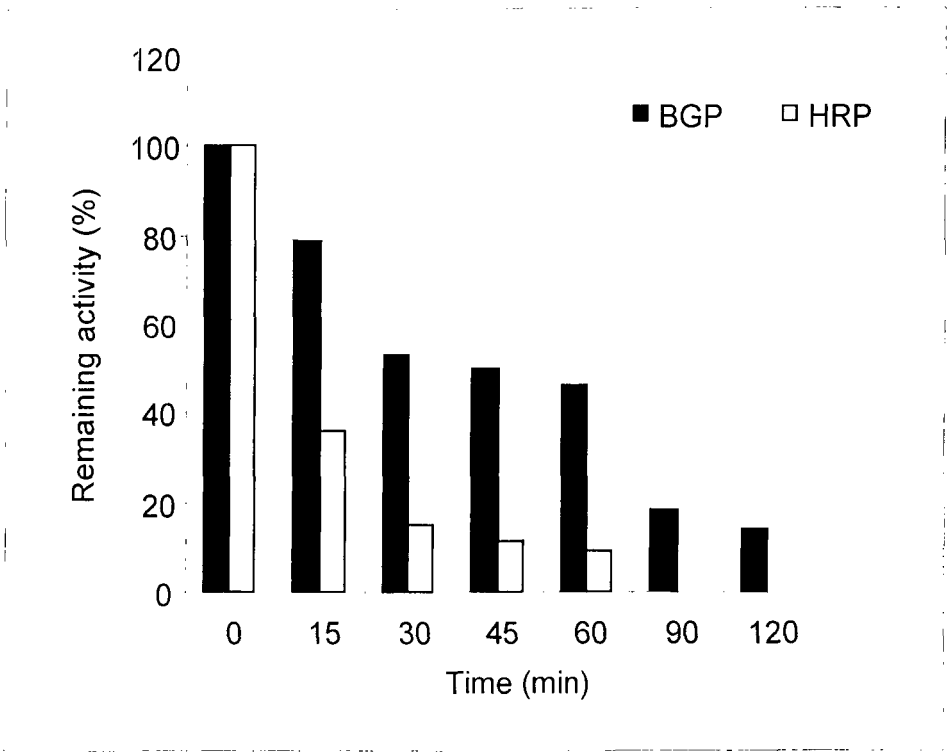


Figure 28: Thermal denaturation of BGP and HRP

BGP and HRP (0.4 U/mL) were incubated at 60 °C for varying times in 100 mM sodium acetate buffer, pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined as described in the text. Unincubated samples at 60 °C were taken as control (100%) for the calculation of percent activity.

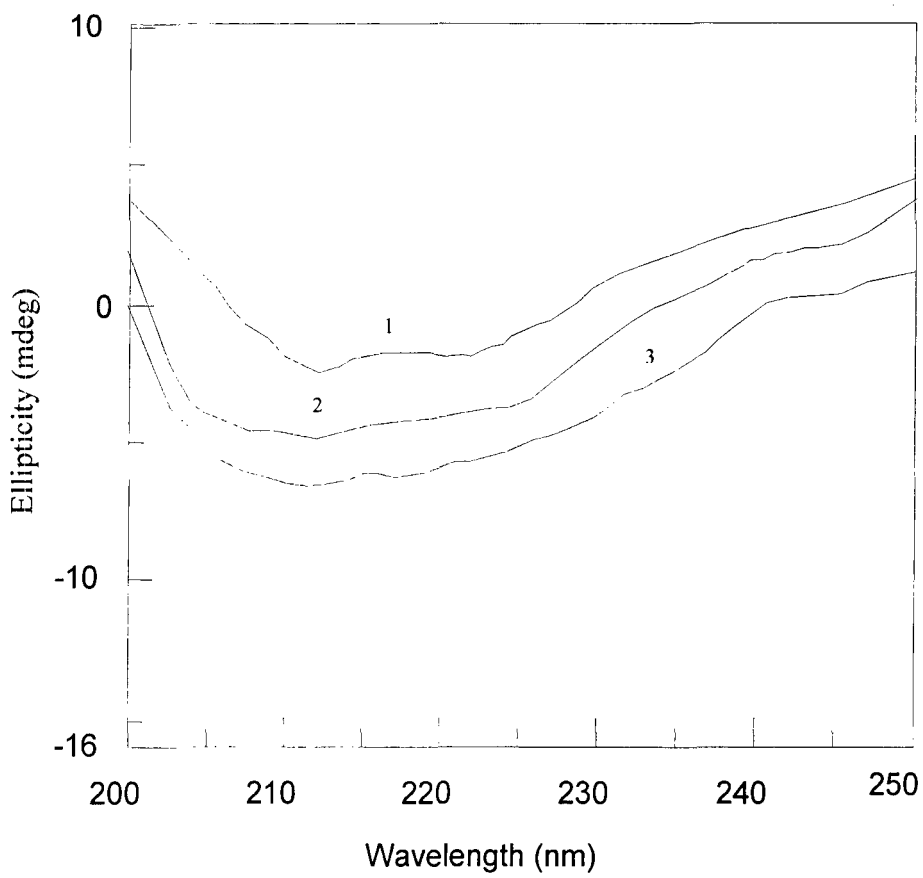


Figure 29: Far UV-CD spectra of BGP at different temperatures

Spectra were taken in the wavelength region of 200-250 nm at three different temperatures (30, 60 and 80 °C). The CD experiment was performed in 100 mM sodium acetate buffer, pH 5.6. The path length of the cell and protein concentration was chosen to optimize the measuring conditions. The spectra are shown at (1) 80 °C, (2) 30 °C and (3) 60 °C.

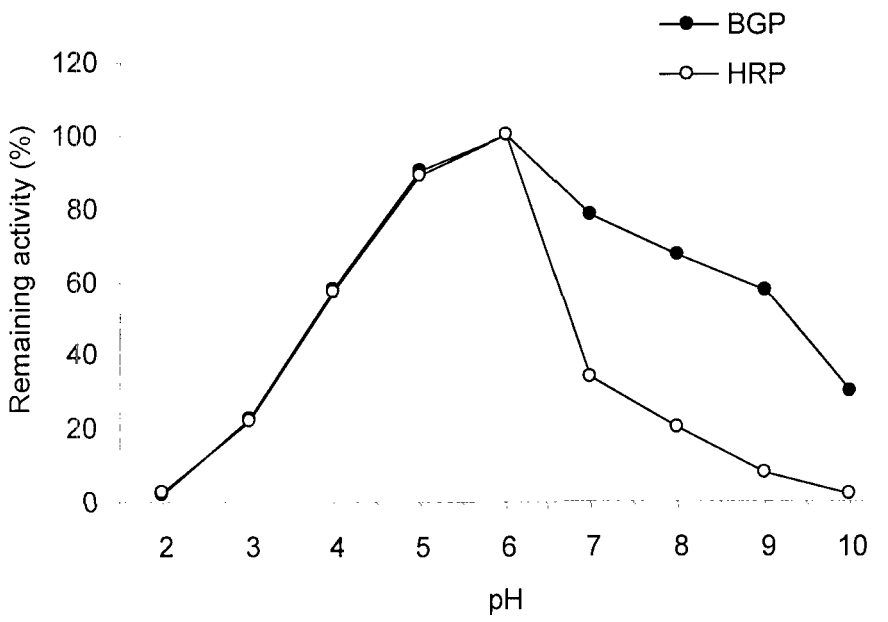


Figure 30: pH-activity profiles of BGP and HRP

BGP and HRP (0.4 U/mL) were assayed in the buffers of different pH values. The buffers used were glycine HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0 and 8.0) and Tris-HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Activity expressed at pH 6.0 was taken as control (100%) for calculating percent remaining activity.

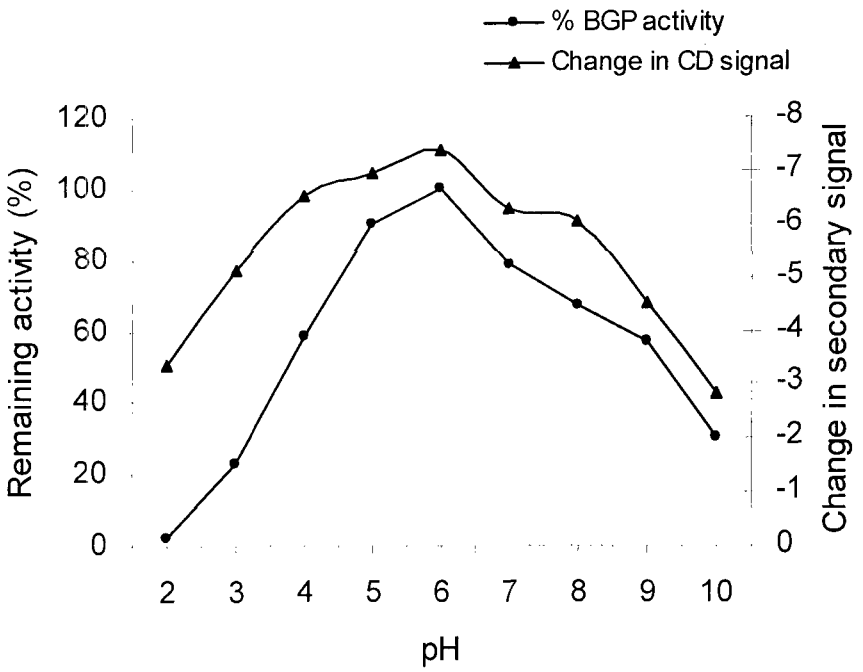


Figure 31: Effect of pH on the activity and secondary structure of BGP

The buffers used were glycine HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0- 6.0), sodium phosphate (pH 7.0 and 8.0) and Tris-HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Activity expressed at pH 6.0 was taken as control (100%) for calculating percent remaining activity. The CD experiment was carried out at 25 °C at 222 nm. The secondary signal has been plotted as mdeg. The protein concentration and path length of the cell were chosen to optimize the measuring conditions.

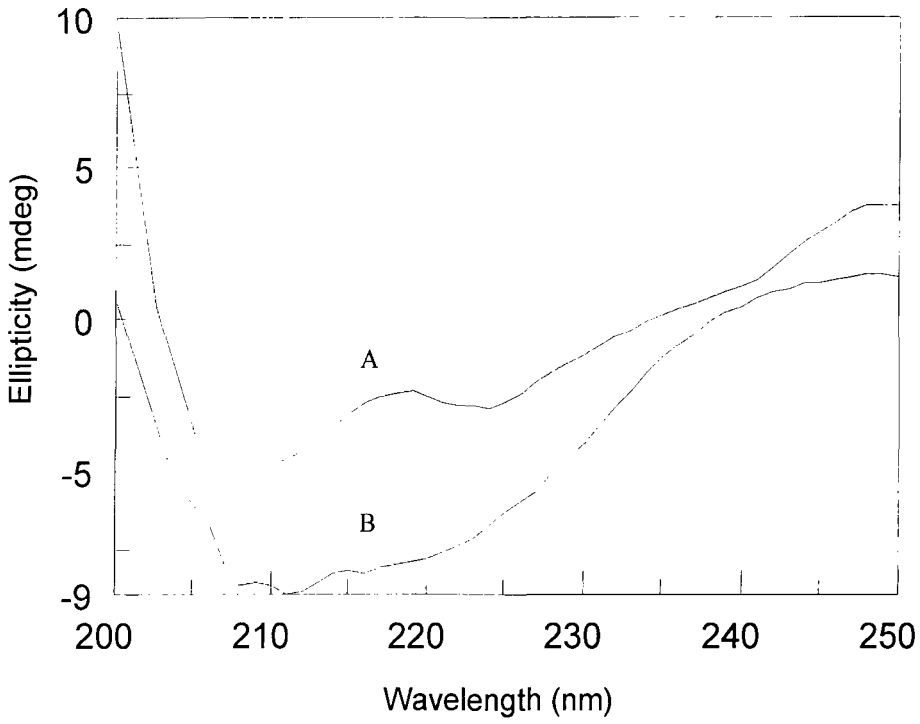


Figure 32: CD spectra of BGP at pH 6.0 and 10.0

The change in the secondary structure of BGP was analyzed at pH 10.0 **(A)** and pH 6.0 **(B)**. CD experiments were carried out at 25 °C. Spectra were taken in the wavelength region, 200-250 nm. Sodium acetate buffer (pH 6.0) and Tris-HCl buffer (pH 10.0) were used in this study. The molarity of each buffer was 100 mM. The protein concentration and path length of the cell were chosen to optimize the measuring conditions.

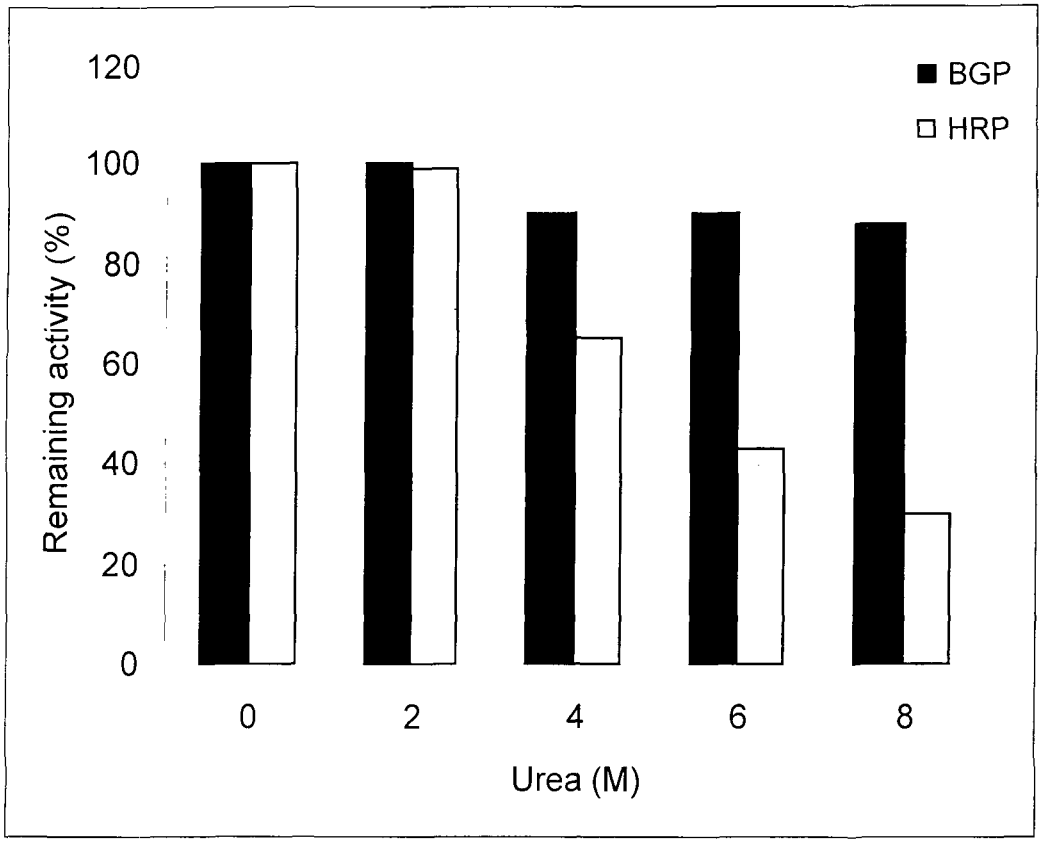


Figure 33: Effect of varying concentration of urea on BGP and HRP

BGP and HRP (0.4 U/mL) were incubated with urea (2.0-8.0 M) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 2 h. Enzyme activity was determined according to the procedure mentioned in the text. For calculating the percent activity, urea untreated enzyme preparations were considered as control (100%).

BGP retained a high activity of 90% after exposure with 6.0 M urea for 2 h whereas HRP lost nearly 50% of the original activity. Further incubation of BGP and HRP with 8.0 M urea resulted in the retention of only 30% activity of HRP whereas BGP exhibited 88% of the initial activity. The stability of both BGP and HRP against the urea (4.0 M) induced inactivation at various time intervals has been investigated (Figure 34). BGP was more resistant to inactivation mediated by 4.0 M urea compared to HRP. HRP retained 64% activity after 2 h exposure with 4.0 M urea whereas BGP retained 90% of the initial enzyme activity under similar treatment.

4.3.4. Effect of SDS

The effect of increasing concentrations of SDS, (0.1-1.0%, w/v) on the activity of BGP and HRP has been shown in Figure 35. There was activation in the activity of both preparations of peroxidase by the exposure of 0.3% (w/v) SDS. BGP activity was remarkably enhanced to 790% whereas HRP exhibited a percent activity of 111% at 0.3% SDS (w/v). The percent enzyme activity of BGP was higher than HRP at all the SDS concentrations and then it leveled off at 0.4% showing a constant value till 1% SDS (w/v). HRP and BGP retained 17 % and 231% of the initial activity at the exposure of 1% SDS for 1 h, respectively.

4.3.5. Effect of organic solvents

Table 7 summarizes the effect of increasing concentrations of water-miscible organic solvents, DMSO and DMF (10-60%, v/v) on the activity of BGP and HRP. There was a conspicuous activation in enzyme activity when treated with increasing concentrations of DMF. However, the activation was more pronounced in case of BGP. BGP was activated to 143% after 1 h incubation in 60% DMF (v/v) at 37 °C whereas HRP was activated to 106% under similar incubation conditions.

The exposure of BGP to varying concentrations of DMSO exhibited an enhancement in its activity. BGP showed 110% of the original activity when exposed to 60% (v/v) DMSO whereas HRP retained only 30% of the enzyme activity under identical experimental conditions (Table 7).

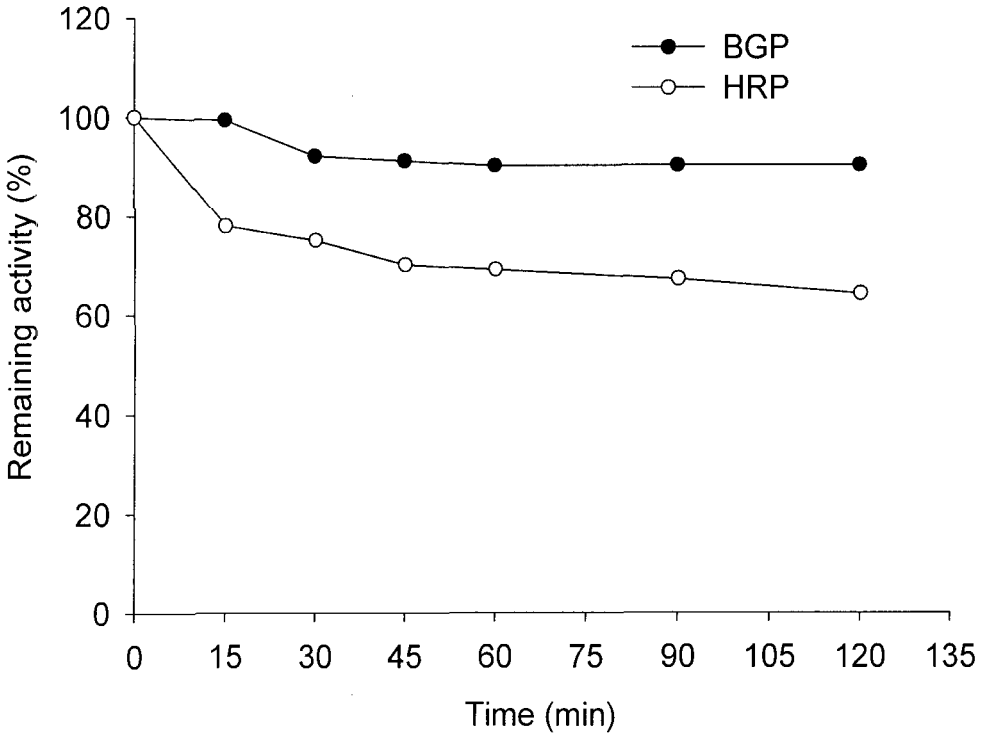


Figure 34: Effect of 4.0 M urea on BGP and HRP

BGP and HRP preparations (0.4 U/mL) were incubated in 4.0 M urea in 100 mM sodium acetate buffer, pH 5.6 at 37 °C. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the percent activity urea untreated samples were considered as control (100%).

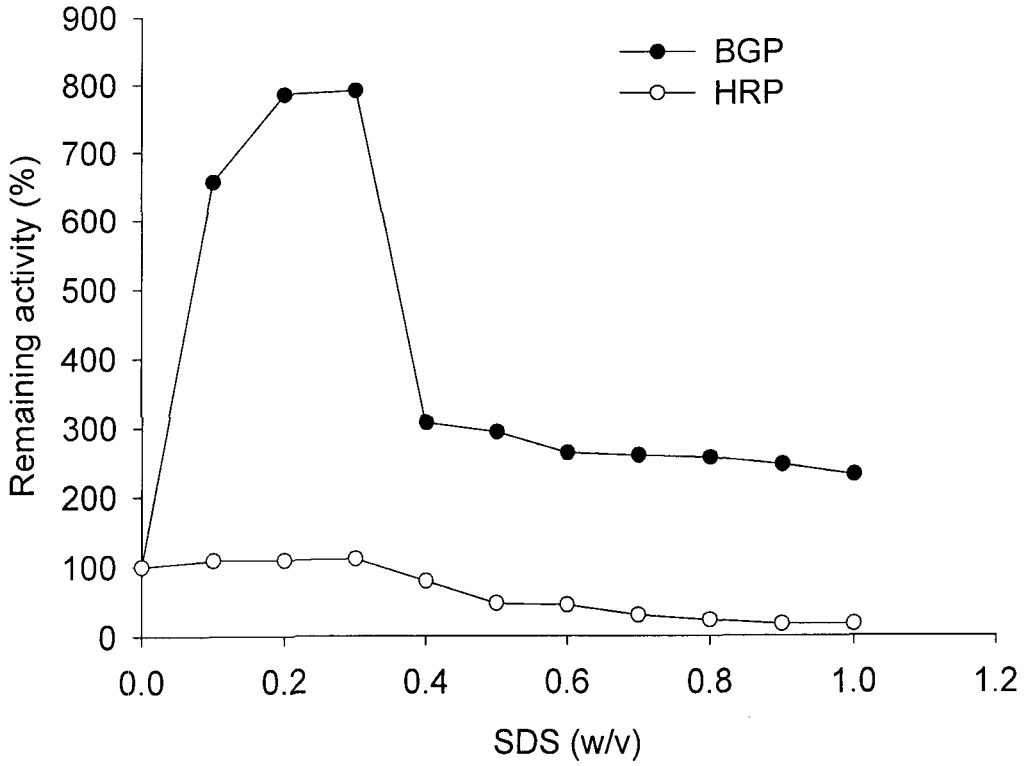


Figure 35: Effect of SDS on BGP and HRP

BGP and HRP (0.4 U/mL) were incubated with increasing concentrations of SDS (0.1-1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. The activity of SDS untreated peroxidase preparations were taken as control (100%) for the calculation of percent remaining activity.

Table 7: Effect of DMF and DMSO on BGP and HRP

Organic solvent (%, v/v)	Percent remaining activity			
	DMF		DMSO	
	BGP	HRP	BGP	HRP
10	89	36	121	91
20	135	103	203	77
30	140	110	210	58
40	143	118	175	55
50	143	106	147	40
60	143	106	110	30

BGP and HRP (0.4 U/mL) were incubated with DMSO and DMF (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. Each value represents the mean for three-independent experiments performed in duplicate, with the average standard deviation not exceeding more than 5%.

4.3. DISCUSSION

Peroxidases have become increasingly important in industries, clinical biochemistry and enzyme immunoassays.

Peroxidases have been reported to be one of the most thermo-stable enzymes in plants (Deepa and Arumughan, 2002). BGP emerged out to be a more thermo-stable enzyme and retained a significantly high fraction of catalytic activity at 60 °C and 80 °C (Figure 25). However, HRP retained relatively less activity at the same temperatures. Earlier investigators have already shown a significant decrease in the catalytic activity of HRP with increase in temperatures (Akita *et al.*, 2001).

Temperature-dependent CD studies of BGP and HRP revealed that BGP retained greater amount of tertiary structure between 30-60 °C as compared to HRP (Figure 26). Moreover, the secondary structure of BGP remained almost stable till 72 °C while HRP lost a substantial secondary structure from 60-80 °C (Figure 27). This observation was further supported by far UV-CD spectra of BGP at 30 °C, 60 °C and 80 °C (Figure 29). It has already been shown that there was a decrease in CD signal intensity of HRP above 60 °C (Akita *et al.*, 2001).

It is evident from thermal denaturation plot of BGP that it retained remarkably higher fraction of catalytic activity after 1 and 2 h of incubation while HRP lost its complete catalytic activity under similar incubation conditions (Figure 28). Some workers have shown that HRP lost its full catalytic activity just after 1 h incubation at 54.7 °C in a buffer of pH 3.0 (Pina *et al.*, 2001).

Several earlier investigators have used CD techniques to study the relationship between the structure and functional stability of proteins (Akita *et al.*, 2001; Regalado *et al.*, 2004). Ellipticity at 222 nm was used to monitor the unfolding of a helical protein (Tsaprailis *et al.*, 1998). It was observed that BGP was far superior in stability than HRP when analyzed through enzymatic assays and CD studies. It has been described that there was a decrease in the stability of HRP with decrease in pH (Pina *et al.*, 2001). However, BGP retained remarkably high fraction of catalytic activity in alkaline pH range (Figure 30). A significant loss in catalytic activity of HRP was further confirmed by low relative activity of HRP at pH 8.0, 9.0 and 10.0 (Kuo and Fridovich, 1988). The retention of higher secondary structure of BGP was further supported by far UV-CD analysis at pH 6.0 and 10.0 (Figure 32). The catalytic

activity of HRP was closely related to its structural changes (Akita *et al.*, 2001; Kamal and Behere, 2003). The change in the catalytic activity of BGP over a wide range of pH values also corresponds to the change in the secondary structure as manifested at 222 nm (Figure 31).

BGP appeared to be more resistant than HRP when both peroxidase preparations were incubated in different concentrations of urea. The catalytic activity of HRP remained unaltered in the presence of 2.0 M urea (Haque *et al.*, 1999). Our observations indicated that there was no change in the enzymatic activity of both BGP and HRP till 2.0 M urea exposure (Figure 33). However, there was a significant decrease in enzyme activity beginning from 4.0 M urea. Haque *et al.* (1999) reported that HRP exposed to 8.0 M urea for 1 h lost 50% of its activity. These results indicated that on exposure to higher concentration of urea (8.0 M) for 2 h, BGP retained 88% activity whereas HRP lost nearly 70% of the original activity.

The activity of BGP was enhanced to 231% at 1.0% SDS (w/v) whereas HRP was activated till a concentration of (0.3%, w/v) and it was inhibited on further exposure to higher concentration of SDS (Figure 35). BGP exhibited a remarkable enhancement in the enzyme activity at 0.3% SDS (w/v). Earlier workers have shown that the activity of some enzymes was enhanced in water/detergent media, owing to the positive interactions between enzyme and detergent (Viparelli and Francesco, 1999). The enhancement in enzyme activity even at high concentration of SDS suggested that BGP could be effectively exploited for various uses in the presence of such detergents for example in reverse micelle bioprocessing applications and for preparation of media for hosting enzyme reactions (Viparelli and Francesco, 1999). Enzymes in the presence of such media could also be exploited in various electrochemical studies (Chattopadhyay and Mazumdar, 2001).

Organic solvents have been used most extensively as solvents for the polymerization of phenols by HRP. Organic solvents are needed for an increase in the solubility of the monomers and for obtaining polyphenols of high molecular weight (Akita *et al.*, 2001). The stability of enzymes is important when organic solvents are used in the media of enzyme catalyzed synthetic reactions. At present, a large body of multienzymatic amperometric biosensors is realized by entrapping peroxidases into a polymer matrix. The polymers used for entrapment are soluble in organic solvents (Santucci *et al.*, 2002). Due to various potential applications of peroxidases in organic

solvents, it became important to investigate the changes in the catalytic activity of both BGP and HRP at different concentrations of organic solvents; DMF and DMSO, (Table 7). Both peroxidases were activated by exposure to 20-60% (v/v) of DMF. Earlier investigators have reported that HRP retained a high catalytic activity in 20-60% (v/v) of DMF (Akita *et al.*, 2001). The catalytic efficiency of enzymatic reactions can be higher in organic media (Lu *et al.*, 1997). These observations indicated that although the activation of HRP took place in 20-60% (v/v) DMF, this activation was low as compared to that of BGP. Kuo and Fridovich (1988) have shown that HRP was activated by those nitrogenous compounds which have a lone pair on nitrogen. This could be a reason for the activation of BGP and HRP by DMF, a nitrogenous compound with a lone pair on nitrogen. These studies further highlighted that when BGP and HRP were treated with increasing concentration of DMSO (10-60%, v/v), BGP activity was remarkably enhanced and it was more stable than HRP. Maeda *et al.* (2002) reported that there was a reduction in catalytic activity of HRP even at low concentrations of DMSO.

Chapter V

Polyclonal antibodies mediated immobilization of ammonium sulphate fractionated bitter gourd peroxidase

5.1. INTODUCTION

Analytical technology based on enzymic biosensors is an extremely broad field which has an impact on many industrial sectors such as the pharmaceutical, healthcare, food and agriculture industries as well as environmental monitoring (Alpeeva *et al.*, 2005; Andreescu *et al.*, 2006., Murphy, 2006). Enzyme immobilization on sensing electrode surfaces is one of the most important points to be considered in biosensor design. The selected procedure of immobilizing enzyme should be able to stabilize the macromolecules and allow easier diffusion of substrates and products to ensure an efficient electron transfer (Ghindilis *et al.*, 1997). However, among immobilization methods being employed; very few can control the spatial distribution of catalyst. Stabilization of enzymes against the inactivation induced by numerous types of denaturants has been accomplished using a multitude of immobilization strategies including covalent coupling, adsorption, microencapsulation, polymer entrapment, chemical aggregation and bioaffinity etc (Husain and Jan, 2000; Duran and Esposito, 2000; Duran *et al.*, 2002).

Procedures that utilize the affinities of biomolecules and ligands for the immobilization of enzymes are gaining wider acceptance in the construction of sensitive enzyme-based analytical devices as well for other applications. The strong affinity of polyclonal/monoclonal antibodies for specific enzymes and those of lectin for glycoenzymes bearing appropriate oligosaccharides have been generally employed for this purpose (Saleemuddin and Husain, 1991; Saleemuddin, 1999; Bucor *et al.*, 2005). Potential of affinity pairs like cellulose-cellulose binding domain bearing enzymes and immobilized metal ion-surface histidine bearing enzymes has also been recognized. Bioaffinity based methods have several advantages over the other known methods used for the immobilization of enzymes (Saleemuddin, 1999). These procedures, in view of their reversibility, lack of chemical modification and the usually accompanying stability enhancement, are emerging as powerful tools for the immobilization of enzymes (Saleemuddin, 1999; Mislovicova *et al.*, 2000; Jan *et al.*, 2001).

Bioaffinity based procedure gives oriented immobilization to enzymes that facilitates good expression of activity and possibility of direct immobilization of enzyme from partially purified preparation or even crude homogenate (Akhtar *et al.*,

2005a; Khan *et al.*, 2005; Kulshrestha and Husain, 2006; Matto and Husain, 2006). Among the bioaffinity pairs the antigen and antibody pair is highly specific and this pair could be exploited for the immobilization of majority of enzymes (Saleemuddin, 1999).

Here an effort has been made to immobilize peroxidase directly from the ammonium sulphate fractionated proteins of bitter gourd on an immunoaffinity support (IgG-Sepharose 4B). The stability of immunoaffinity immobilized BGP has been investigated against heat, pH, chaotropic agents (urea and guanidinium chloride), detergents (CTAB and Surf Excel), proteolytic enzyme (trypsin) and water-miscible organic solvents (propanol, THF and dioxane). Immobilized BGP preparation was also compared for its stability with its soluble form.

5.2. MATERIALS & METHODS

5.2.1. Materials

Sepharose 4B, Sephacryl S-100, trypsin, cyanogen bromide (CNBr), DEAE-cellulose, GdnCl and reagents for electrophoresis were purchased from Sigma Chem. Co. (St. Louis, MO.) USA. Con A-Sepharose was the product of Genei, India. All other chemicals and reagents used were of analytical grade. Bitter gourd was obtained from the local market.

5.2.2. Immunization

Glycosylated form of BGP was isolated to homogeneity by using a three-step purification scheme. The detailed procedure for the isolation of this enzyme has already been described in Chapter II. Purified glycosylated BGP was injected into healthy male albino rabbits weighing 2-3 kg for the production of anti BGP polyclonal antibodies. The animals received subcutaneously 300 μg of BGP dissolved in 0.5 mL of 20 mM sodium phosphate buffer, pH 7.2, mixed and emulsified with equal volume of Freund's complete adjuvant as first dose (Jan *et al.*, 2001). Boosters' doses of 150 μg of BGP mixed and emulsified with Freund's incomplete adjuvant

were administered weekly after resting the animal for 15 days. After each booster dose blood was collected from the ear vein of the animal and allowed to clot at room temperature for 3 h. Serum was collected by centrifugation at 1600 *g* for 20 min at 4 °C and later it was decomplimented by incubating at 56 °C for 30 min. After adding sodium azide (0.2%) serum was stored at -20 °C.

5.2.3. Purification and characterization of polyclonal antibodies

The antiserum was fractionated with 20-40% ammonium sulphate. The sample was kept overnight with constant stirring at 4 °C to precipitate out proteins. The precipitated proteins were collected by centrifugation at 1600 *g* for 20 min at 4 °C. The pellet obtained was re-dissolved in a minimum volume of 20 mM sodium phosphate buffer, pH 7.2 and was subjected to extensive dialysis against the same buffer to remove traces of ammonium sulphate.

Antibodies against BGP were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate precipitated antiserum was passed through DEAE-cellulose column (1.20x10.0 cm) and the fractions containing purified antiperoxidase antibodies were pooled for further use (Khan *et al.*, 2005).

SDS-PAGE on 12.5% gel under denaturing conditions and native PAGE on 7.5% gel were run to separate proteins present in DEAE-cellulose purified antiperoxidase antibodies, according to the procedure described by Laemmli (1970). The staining and de-staining was also performed by the same procedure. M_r marker proteins (myosin, 205 kDa; β galactosidase, 116 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa and lysozyme, 14.3 kDa) were also run in one lane adjacent to the purified IgG. The M_r of IgG subunits was calculated by plotting the mobility of marker proteins vs the logarithm of their molecular weights.

5.2.4. Immunodiffusion

Ouchterlony double immunodiffusion was used to confirm the presence of antibodies against BGP. Immunodiffusion was performed in 1.0% (w/v) agarose

prepared in normal saline (Jan *et al.*, 2001). The cross-reactivity of antibodies was also checked against HRP. The purified antiperoxidase antibodies were employed for preparing immunoaffinity support.

5.2.5. Direct binding ELISA

Polystyrene (96 well) microtitre plate was coated with 100 µL of antigen (BGP) at a concentration of 10 µg/mL prepared in antigen coating buffer (50 mM bicarbonate buffer, pH 9.6) and then incubated for 2 h at 37 °C followed by overnight storage at 4 °C. The wells were then washed three-times with TBS-T buffer. The unoccupied sites were blocked with 2% fat milk in TBS (150 µL each well) followed by incubation for 5-6 h at room temperature. The wells were then washed twice with TBS-T buffer. The test and control wells were then diluted with 100 µL of serially diluted serum. Each dilution was in TBS buffer. Serially diluted blanks corresponding to each dilution were also present. The plate was then incubated for 2 h at room temperature and overnight at 4 °C. The plate was washed again with TBS-T buffer (five times). Bound antibodies were assayed with an appropriate conjugate of anti rabbit alkaline phosphatase (1:3000), 100 µL of it was coated in each well and kept at room temperature for 2 h. Washing of the plate with TBS-T (five times) and with distilled water (two times) was followed by addition of p-nitrophenyl phosphate (50 µg/100 µL) in each well and incubation at 37 °C for 30-45 min. The absorbance of each well was monitored at 405 nm on a Lab System ELISA Reader.

5.2.6. Preparation of immunoaffinity support

Sepharose 4B (5.0 g) was activated as described by Porath *et al.* (1967). The Sepharose 4B was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 10.0 mL of 1.0 M Na_2CO_3 and stirred slowly by placing on a magnetic stirrer at 4 °C for 30 min. CNBr (1.0 g) dissolved in 1.0 mL of acetonitrile was added to the beaker containing Sepharose 4B and was again stirred for 10 min in cold. The whole mass was transferred immediately to a sintered funnel and washed thoroughly with sufficient volume of 0.1 M bicarbonate

buffer, pH 8.5, distilled water, and again with same buffer. Washed and activated Sepharose 4B was dried and re-suspended in 5.0 mL of 0.1 M bicarbonate buffer, pH 8.5. Purified antibodies (60 mg) were mixed with 5.0 mL of activated Sepharose 4B and stirred overnight at 4 °C. Sepharose bound antibodies was centrifuged to remove the unbound antibodies. Antibody bound matrix was extensively washed with 0.1 M bicarbonate buffer, pH 8.5 containing 1.0 M NaCl. This washed suspension was treated with 7.0 mL of 0.1 M glycine for 2 h at 4 °C. Antibody bound matrix was successively washed with 0.1 M sodium bicarbonate buffer, pH 8.5 containing 1.0 M NaCl, distilled water and finally with 50 mM sodium phosphate buffer, pH 7.0. The quantity of bound antibody was calculated by subtracting the unbound protein in the washings from that of the total added protein.

5.2.7. Ammonium sulphate fractionation of bitter gourd proteins

Ammonium sulphate fractionation of bitter gourd proteins was carried out according to the procedure described in Chapter II.

5.2.8. Purification of BGP by gel filtration and bioaffinity chromatography

The purification procedure of BGP has been described in detail in Chapter II.

5.2.9. Immobilization of BGP on IgG-Sepharose 4B

Ammonium sulphate fractionated and dialyzed enzyme solution (4 mL, 2925 U BGP) was mixed with 4.0 mL of IgG-Sepharose 4B. The mixture was stirred overnight at 4 °C. The gel was then thoroughly washed with 50 mM sodium phosphate buffer, pH 7.0 to remove unbound enzyme (Jan and Husain, 2004).

5.2.10. Effectiveness factor (η)

The effectiveness factor (η) value of the immobilized preparation represents the ratio of actual and theoretical activity of the immobilized enzyme (Muller and Zwing, 1982).

5.2.11. Measurement of peroxidase activity

Peroxidase activity was estimated according to the procedure described in Chapter II.

5.2.12. Estimation of Protein concentration

Protein was estimated by the method of Lowry *et al.* (1951). Aliquots of protein were taken in a set of tubes and the final volume was made up to 1 mL with distilled water. Five milliliter of alkaline copper reagent (containing one part of 1% (w/v) copper sulphate and 2% (w/v) sodium potassium tartarate in 1% (w/v) sodium hydroxide and sodium carbonate) was added. After 10 min of incubation at room temperature 0.5 mL of 1.0 N Folin-Ciocalteu's phenol reagent was added to the tubes. The tubes were instantly vortexed. The color developed was read at 660 nm after 30 min against a reagent blank. A standard curve was prepared using bovine serum albumin.

5.2.13. Effect of temperature

Soluble and immobilized BGP (0.4 U/mL) were incubated in 100 mM sodium acetate buffer, pH 5.6 at 60 ^{o}C for varying time intervals. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and then the peroxidase activity was determined.

5.2.14. Effect of urea

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of urea (2.0-8.0 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 ^{o}C. Peroxidase activity was determined after each incubation period. The activity

of urea untreated enzyme was considered as control (100%) for calculating percent activity.

5.2.15. Effect of GdnCl

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of GdnCl (0-3.5 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 °C. Peroxidase activity was determined after each incubation period. The activity of the GdnCl untreated enzyme was considered as control (100%) for calculating percent activity.

5.2.16. Effect of trypsin

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of trypsin (0.25-2.5 mg/mL) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. For calculating the percent activity trypsin untreated samples were considered as control (100%).

5.2.17. Effect of organic solvents

Soluble and immobilized BGP were incubated with water-miscible organic solvents; propanol/THF/dioxane (0-60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of soluble and immobilized BGP in assay buffer without any water-miscible organic solvent was taken for the calculation of percent activity as control (100%). Peroxidase activity was assayed at all the indicated organic solvent concentrations.

5.2.18. Effect of detergents

Soluble and immobilized BGP (0.4 U/mL) were incubated with CTAB and Surf Excel (0.2-2.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of soluble and immobilized BGP in assay buffer without any detergent

was taken as control (100%) for the calculation of percent activity. Peroxidase activity was determined at all the indicated detergent concentrations.

5.3. RESULTS

5.3.1. Production and purification of anti BGP polyclonal antibodies.

BGP purified to homogeneity was highly immunogenic in rabbits. Purified antibodies raised against BGP gave a clear single precipitin line with the enzyme as evident from (Figure 11, Chapter II). There was a cross reaction with HRP (data not given). The glycan structures on plant glycoproteins (including peroxidases) are highly antigenic (van Huystee and McManus, 1998). These findings suggested that the peroxidase was immunogenic in rabbits. DEAE-cellulose purified IgG loaded on SDS-PAGE clearly showed two bands corresponding to the heavy and light chains of the antibody (Figure 36a). The position of migration of IgG subunits correspond to apparent molecular weights of 45 kDa and 20 kDa (Figure 36b). The native PAGE showed a single band (Figure 37) and this result supported the purity of the purified antibodies. The titer obtained through direct binding ELISA was greater or equal to 51200 (Figure 38). Overnight incubation of fixed amount of BGP with increasing concentration of purified IgG exhibited no loss in enzyme activity. These observations further supported that the antiserum raised against the purified BGP contained only non-inhibitory antibodies (data not given).

5.3.2. Immobilization of BGP on the anti BGP IgG-Sepharose 4B

The IgG isolated by ammonium sulphate fractionation and ion exchange chromatography were used for the construction of Sepharose 4B-anti BGP-immunoaffinity support for the immobilization of BGP from the partially purified BGP preparation.

Figure 36a: Separation of anti BGP polyclonal antibodies and marker proteins by SDS-PAGE.

SDS-PAGE (12.5%) was used to characterize the purified antibodies.

DEAE fraction. 30 µg was loaded in the well.

Lane A: Marker Proteins

Lane B: Purified anti BGP polyclonal antibodies

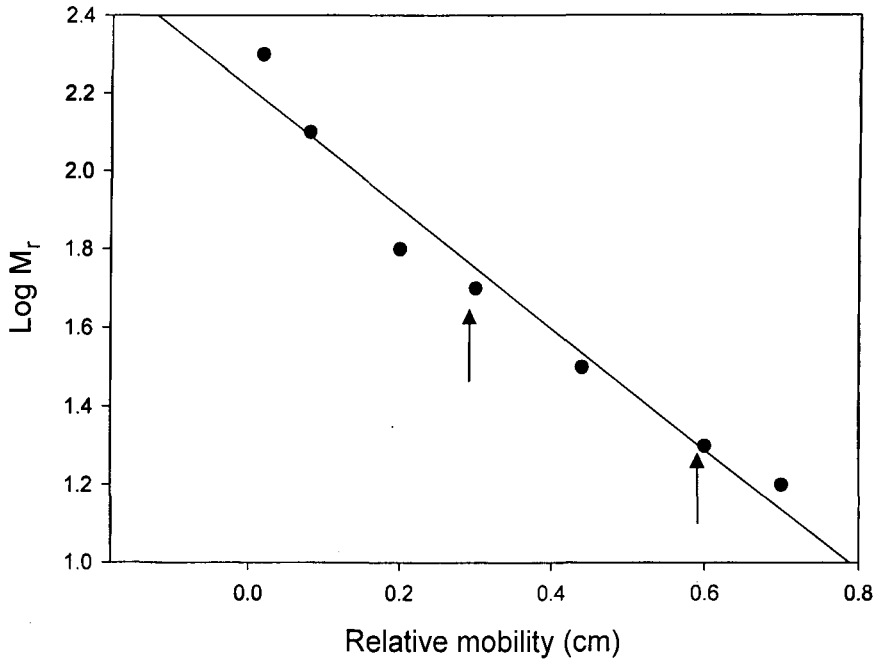


Figure 36b: Molecular weight vs relative mobility plot

The relative mobility of the standard marker proteins from the SDS gel (Figure 36a) were plotted against logarithm of M_r using least square analysis. Arrow indicates the position of the large and small M_r peptides from DEAE fractions.

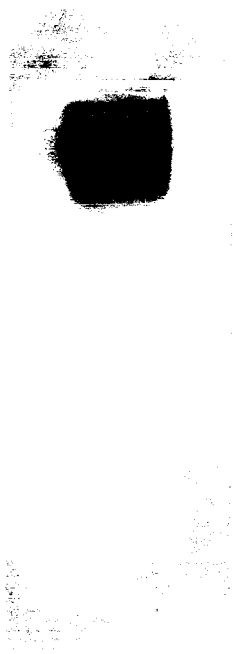


Figure 37: Native PAGE for purified anti BGP polyclonal antibodies.

Non-denaturing, native PAGE (7.5%) was run. DEAE cellulose eluted fraction containing 30 μg was loaded in the well.

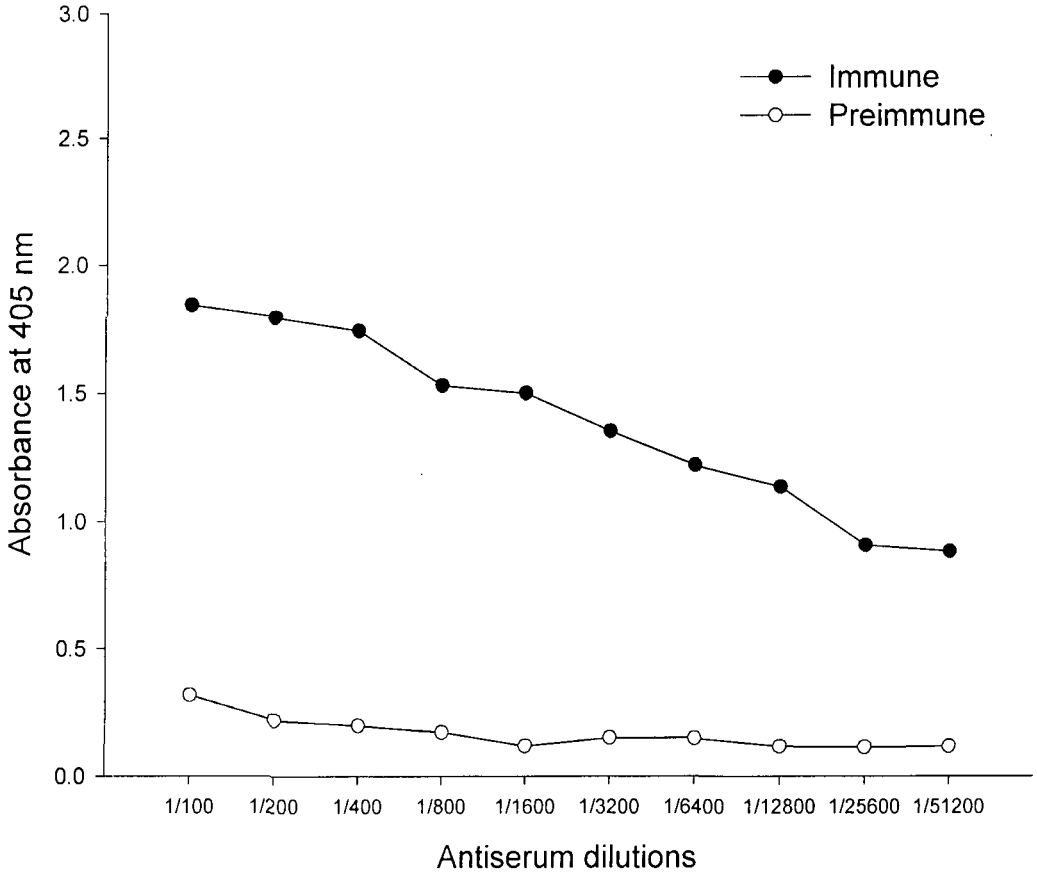


Figure 38: Direct binding ELISA

Serially diluted antiserum and preimmune serum were incubated in a polystyrene microtitre plate with the antigen (BGP). Serially diluted blanks corresponding to each dilution were also present. The absorbance of each well (after addition of substrate, p-nitrophenyl phosphate) was monitored at 405 nm on Lab Systems ELISA Reader.

Sepharose 4B bound 9.69 mg of IgG per mL of the gel. Anti BGP polyclonal antibody bound Sepharose 4B specifically retained nearly 615 U of peroxidase/mL of the matrix. The preparation thus obtained was highly active and exhibited very high effectiveness factor 'η' as 0.96 (Table 8).

5.3.3. Stability properties of soluble and immobilized BGP

In order to monitor the compatibility of immobilized BGP preparation in various applications, the investigations of its stability against different physical and chemical parameters is necessary. The thermal stability of soluble and immunoaffinity bound BGP was monitored after incubating at 60 °C for various time intervals (Figure 39). Immobilized BGP exhibited nearly 44% of the original activity even after 2 h incubation at 60 °C while the soluble enzyme lost nearly 83% activity under similar experimental conditions. Immunoaffinity bound BGP exhibited a marginal broadening in temperature-activity profile, there being no difference in activity between 30-40 °C whereas free BGP had a temperature-optima at 40 °C. Immunoaffinity bound BGP retained greater fractions of catalytic activity on both sides of temperature-optima compared to free enzyme (Figure 40).

Both soluble and immobilized BGP exhibited activity peak at pH, 6.0 (Figure 41). Immobilized enzyme retained significantly higher enzyme activity on both sides of pH-optima in comparison to free enzyme.

The effect of different concentrations of urea on soluble and immobilized BGP is depicted in Figure 42. Immobilized enzyme did not lose any activity till 2.0 M urea exposure. At 8.0 M urea the soluble enzyme retained 86% of the activity whereas the immobilized enzyme preparation exhibited 93% of the initial activity. Figure 43 demonstrates the urea-induced inactivation of soluble and immobilized BGP. IgG-Sepharose bound BGP retained about 95% of the original enzyme activity after exposure to 4.0 M urea for 2 h whereas its soluble counterpart retained 83% activity.

Effect of GdnCl concentrations on both soluble and immobilized BGP is shown in Figure 44.

Table 8: Immobilization of BGP on IgG-Sepharose 4B support

Amount of enzyme loaded (X) (U)	Amount of enzyme activity in washes (Y) (U)	Activity bound /mL of IgG-Sepharose 4B (U)			% Activity yield (B/Ax100)
		Theoretical (X-Y=A) (A)	Actual (B)	Effectiveness factor (η) (B/A)	
731	90	641	615	0.96	96

Each value represents the mean for three-independent experiments performed in duplicate. The standard deviation was not exceeding more than 5%. Peroxidase activity was assayed according to the procedure described in the text.

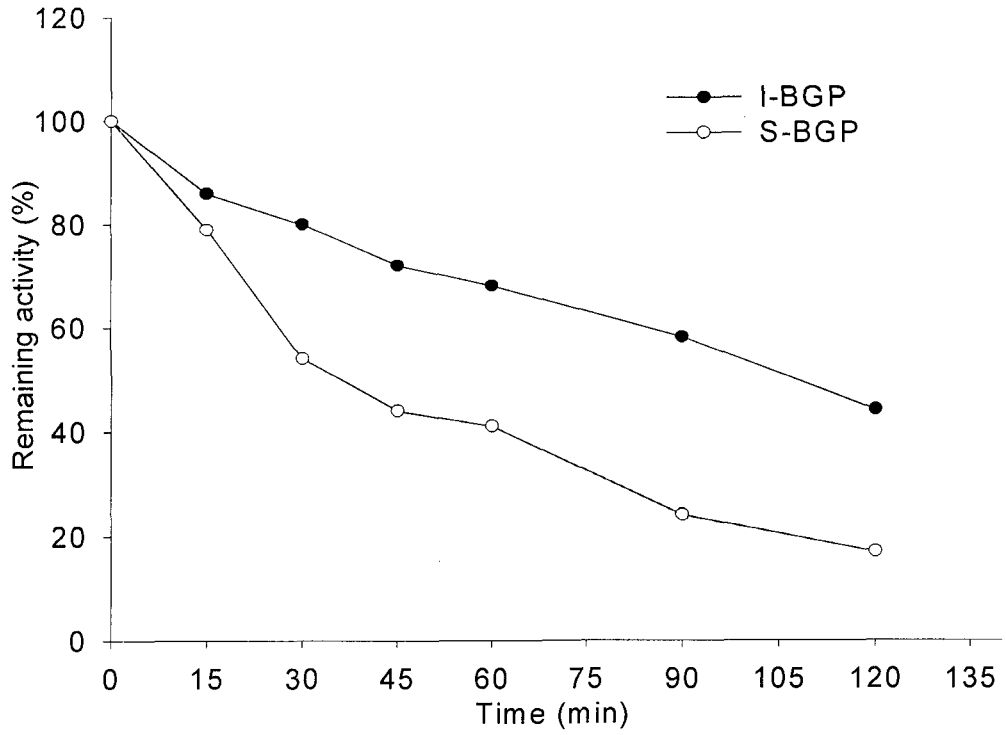


Figure 39: Thermal denaturation of soluble and immobilized BGP.

The appropriate amount of soluble and immobilized BGP preparations were incubated at 60 °C for various time intervals in 100 mM sodium acetate buffer, pH 5.6. Aliquots of appropriate and equal amount were removed at different time intervals and the enzyme activity was determined as described in the text. Unincubated samples at 60 °C were taken as control (100%) for calculation of percent activity.

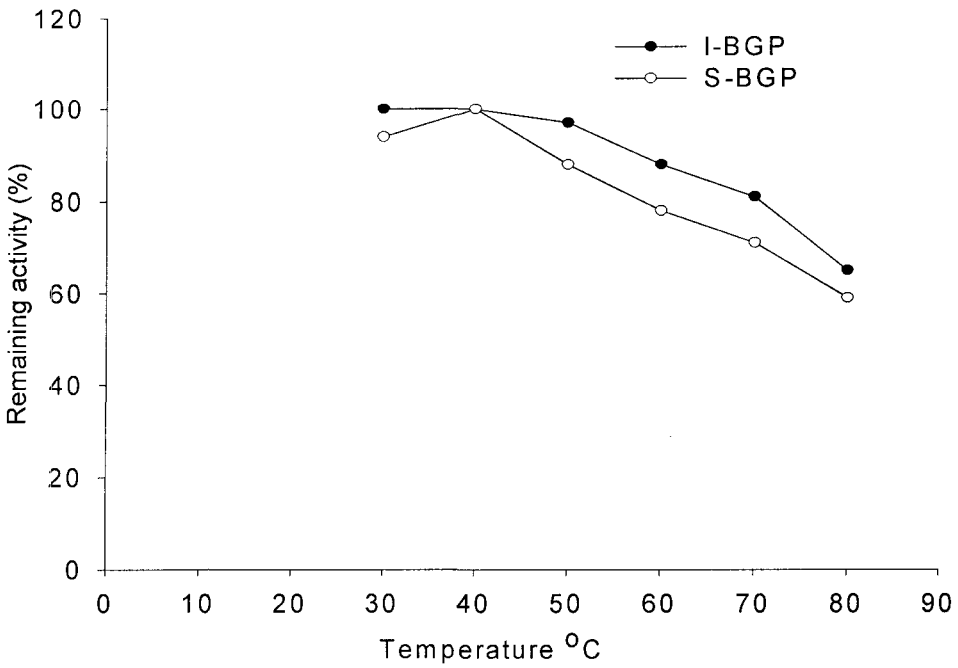


Figure 40: Temperature-activity profiles of soluble and immobilized BGP

The activity of soluble and immobilized BGP (0.4 U/mL) was monitored at various indicated temperatures. Activity expressed at 40 °C was taken as control for calculating percent activity.

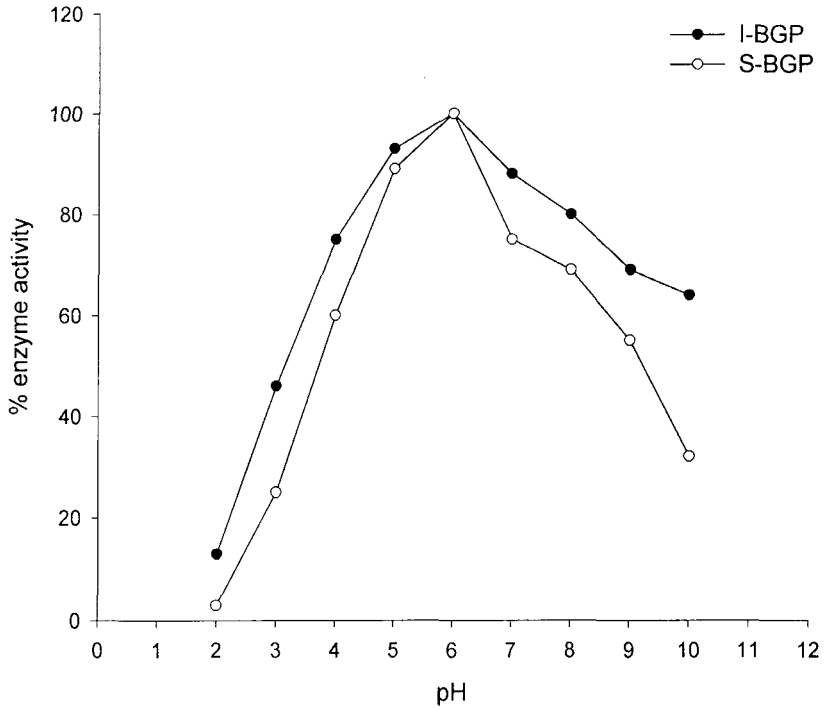


Figure 41: pH-activity profiles of soluble and immobilized BGP.

Soluble and immobilized BGP 0.4 U/mL were taken for assaying the activity of the enzyme preparations in the buffers of varying pH values. The reaction mixture was incubated at 37 °C for 15 min in buffers having pH range from 2.0 to 10.0. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0 and 8.0) and Tris HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. The percent remaining enzyme activity was calculated by taking activity at optimum pH as control (100%).

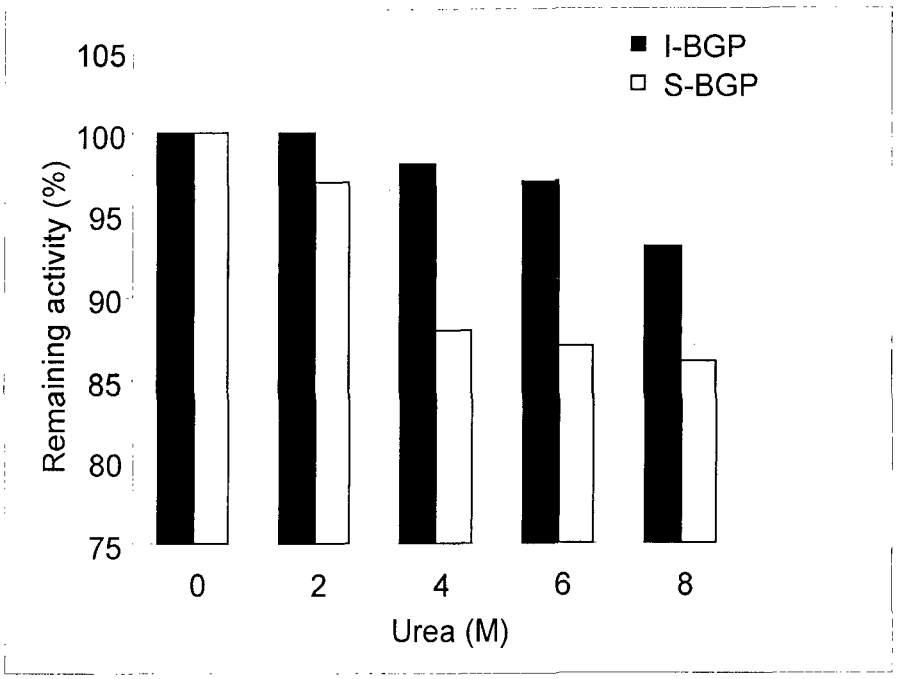


Figure 42: Effect of urea on soluble and immobilized BGP

Soluble and immobilized BGP (0.4 U/mL) were incubated in varying concentrations of urea (2.0-8.0 M) prepared in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 2 h. Enzyme activity was determined according to the procedure mentioned in the text. For calculating the percent activity urea untreated BGP preparations were considered as control (100%).

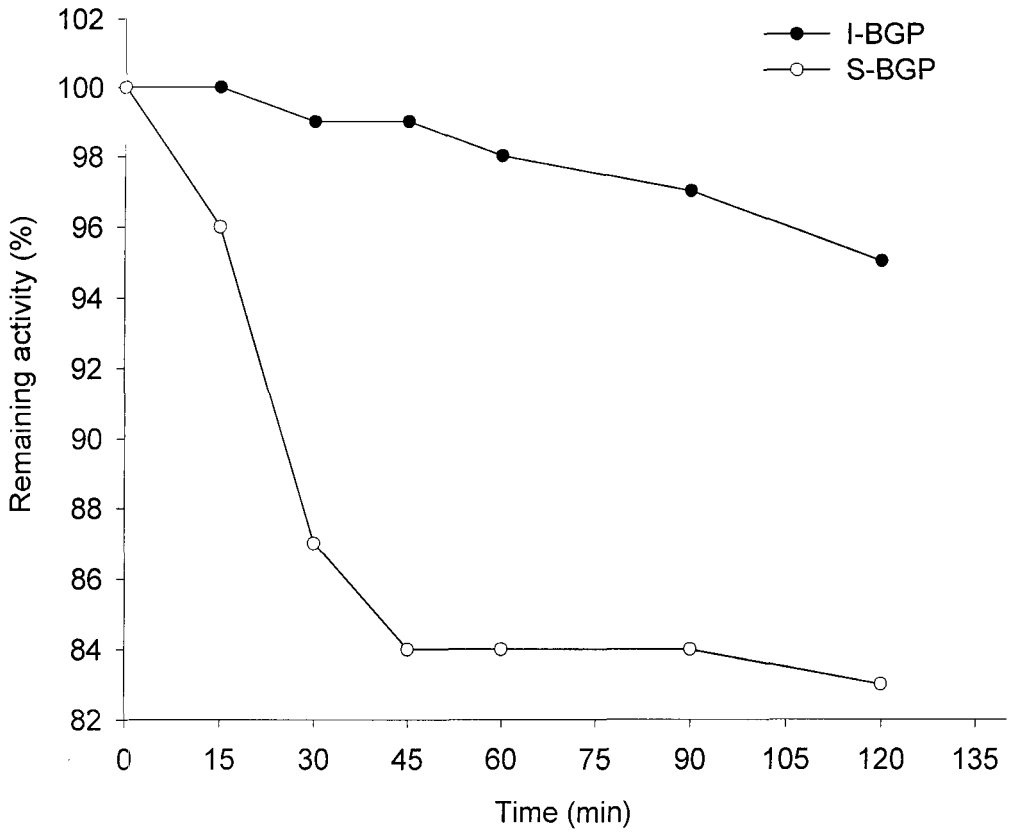


Figure 43: Effect of 4.0 M urea on soluble and immobilized BGP

Soluble and immobilized BGP (0.4 U/mL) was incubated with 4.0 M urea in 100 mM sodium acetate buffer, pH 5.6. Aliquots of appropriate amount of each preparation were taken at various time intervals and activity was determined by procedure given in the text. For calculating the percent activity urea untreated BGP preparations were considered as control (100%).

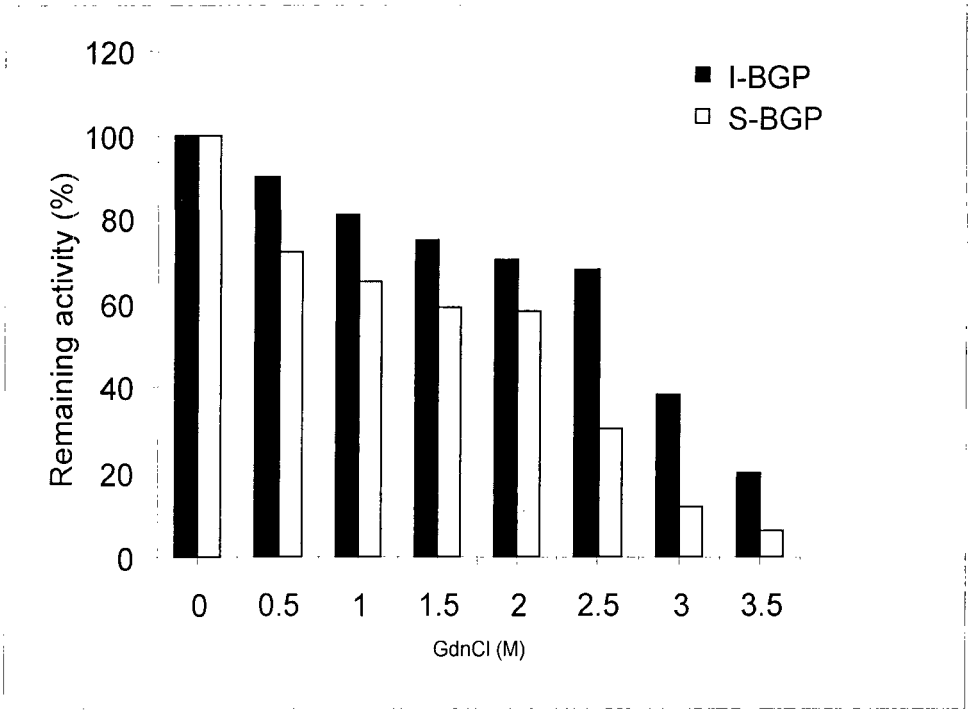


Figure 44: Effect of GdnCl on soluble and immobilized BGP

Soluble and immobilized BGP (0.4 U/mL) were incubated in 100 mM sodium acetate buffer, pH 5.6 containing (0-3.5 M) GdnCl at 37 °C for 1 h. Enzyme activity was determined as mentioned in the text. For calculating the percent activity GdnCl untreated BGP preparations were considered as control (100%).

Immobilized enzyme retained 38% activity at 3.0 M GdnCl whereas soluble enzyme exhibited only 12% of the initial enzyme activity. Immobilized enzyme preparation was more stable than its soluble form at all the concentrations indicated. The effect of 2.0 M GdnCl at different time intervals is indicated in Figure 45. Immobilized enzyme retained nearly 50% activity after 2 h of incubation whereas soluble counterpart lost nearly 70% of its original activity under similar incubation conditions.

Soluble and immobilized BGP were treated with increasing concentrations of trypsin (0.25-2.5 mg/mL, w/v) for 1 h at 37 °C (Figure 46). The activity of immobilized BGP enhanced to 120% after exposure to 1.0 mg/mL trypsin whereas the soluble enzyme exhibited a loss of 10% of the initial activity under identical experimental conditions. At 2.5 mg/mL trypsin exposure immobilized BGP preparation retained 93% activity whereas the soluble enzyme lost 50% of the initial activity under similar treatment. The immobilized BGP showed greater stability at almost all the concentrations of trypsin.

The soluble and immunoaffinity bound BGP preparations were treated with various concentrations of water-miscible organic solvents (0-60%, v/v) for 1 h at 37 °C (Table 9). Immobilized BGP retained 69% of the original activity when it was exposed to 30% (v/v) propanol for 1 h while its soluble counterpart exhibited 44% of its initial activity under identical treatment. Exposure of IgG-Sepharose 4B bound BGP to higher concentrations of propanol resulted in the retention of more than 50% activity whereas the free enzyme showed a rapid loss in catalytic activity. The latter retained only 28% activity at 60% (v/v) of propanol. The immobilized BGP after treatment with 30% (v/v) THF retained 86% of the original activity; however the soluble BGP under similar exposure showed only 65% of its activity. More than 50% of the original activity was retained by immobilized BGP after treatment with 60% (v/v) dioxane while the soluble enzyme lost nearly 80% of its activity at the same concentration of dioxane.

The effect of different detergents (0.2-2.0%, w/v) on activity of both preparations of BGP is shown in Table 10. In the presence of CTAB both soluble and immobilized BGP showed an enhancement in their catalytic activity. However, the enhancement in the activity for the immobilized preparation was higher than the soluble form of the enzyme at each concentration of CTAB.

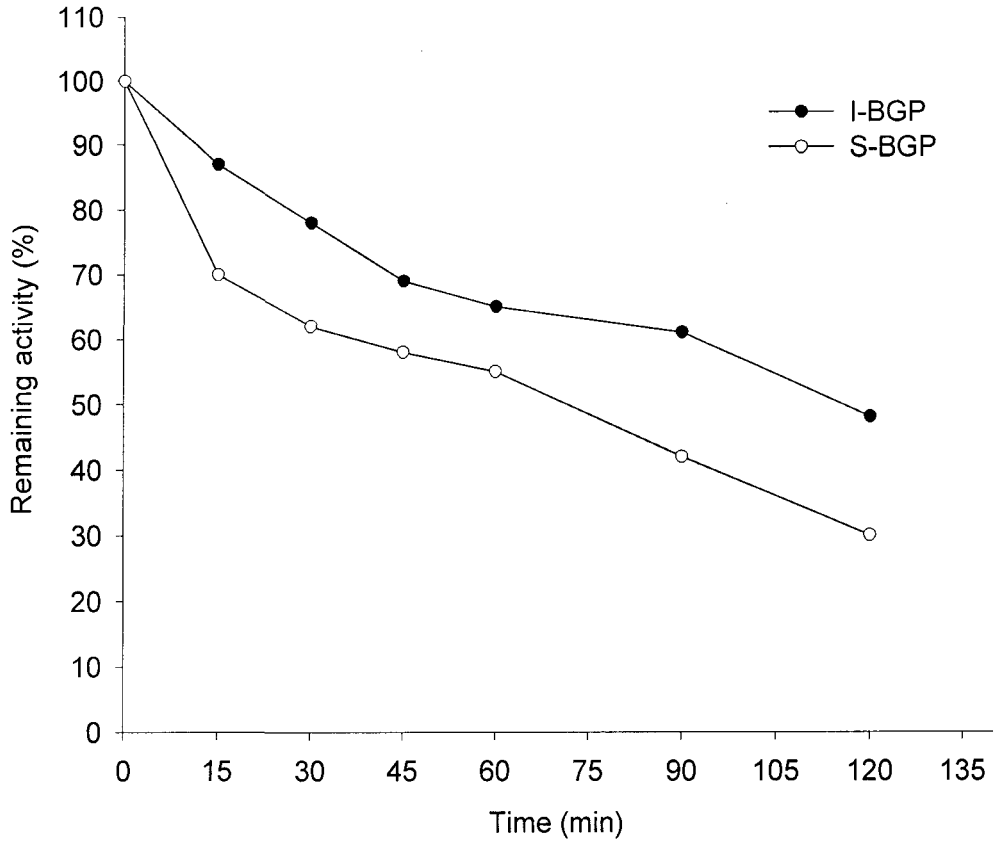


Figure 45: Effect of 2.0 M GdnCl on soluble and immobilized BGP

Soluble and immobilized BGP (0.4 U/mL) were incubated with 2.0 M GdnCl in 100 mM sodium acetate buffer, pH 5.6. Aliquots of appropriate amount of each preparation were taken at various time intervals and activity was determined by procedure given in the text. For calculating the percent activity GdnCl untreated BGP preparations were considered as control (100%).

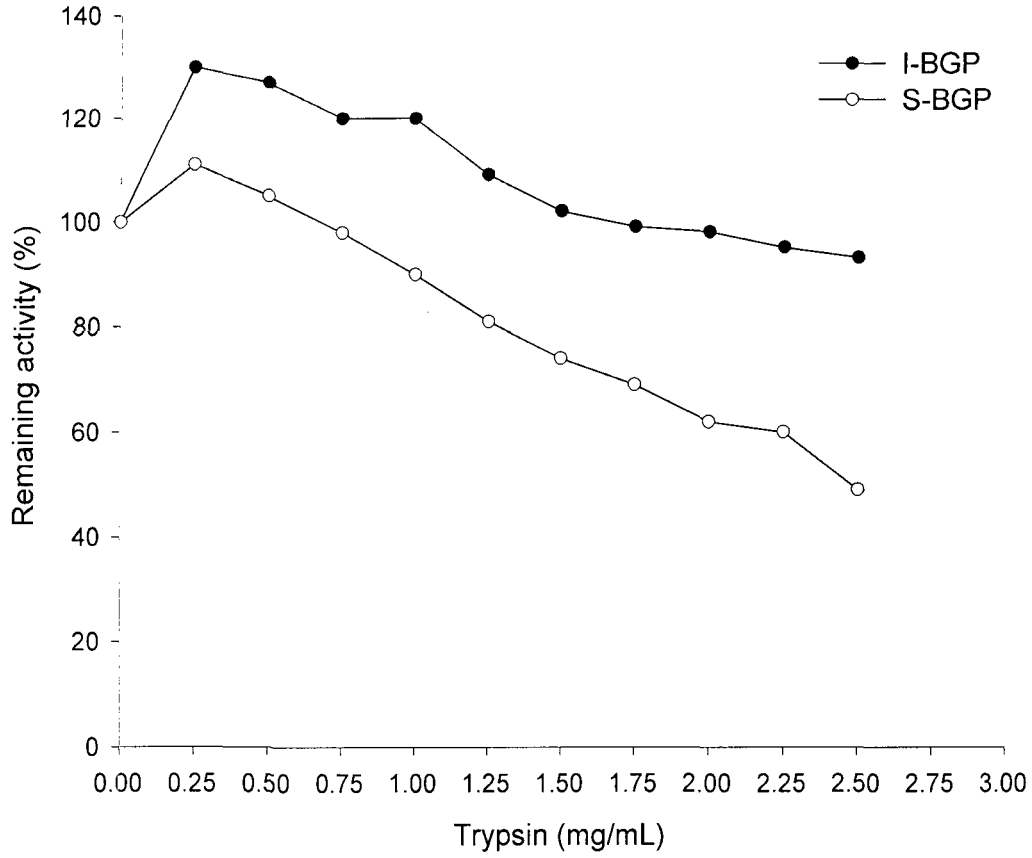


Figure 46: Effect of trypsin on soluble and immobilized BGP

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of trypsin (0.25-2.5 mg/mL) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. For calculating the percent activity trypsin untreated BGP preparations were considered as control (100%).

Table 9: Effect of water miscible organic solvents on soluble and immobilized BGP

Organic solvent %	Percent remaining activity					
	Propanol		THF		Dioxane	
	S-BGP	I-BGP	S-BGP	I-BGP	S-BGP	I-BGP
0	100	100	100	100	100	100
10	69	82	80	93	64	85
20	57	79	70	90	59	81
30	44	69	65	86	52	71
40	39	60	58	78	41	70
50	34	58	50	65	32	68
60	28	52	45	60	20	64

Soluble and immobilized BGP were incubated with propanol/THF/dioxane 0-60% (v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any water-miscible organic solvent was taken for the calculation of percent activity as control (100%) separately. Each value represents the mean for three independent experiments performed in duplicate with the average standard deviation not exceeding more than 5%.

Table 10: Effect of detergents on soluble and immobilized BGP

Detergent (%, v/v)	Percent remaining activity			
	CTAB		Surf Excel	
	S-BGP	I-BGP	S-BGP	I-BGP
0.2	149	178	103	111
0.4	142	178	90	102
0.6	130	159	82	98
0.8	125	157	70	95
1.0	119	150	62	91
1.2	116	148	50	77
1.4	112	140	48	70
1.6	109	137	36	58
1.8	105	133	27	50
2.0	104	133	12	48

Soluble and immobilized BGP (0.4 U/mL) were incubated with CTAB and Surf Excel (0.2-2.0 %, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined in all the tubes as described in the text. The activity of soluble and immobilized BGP without detergent treatment was considered as control (100%) for the calculation of per cent activity. Each value represents the mean for three-independent experiments performed in duplicate with the average standard deviation not exceeding more than 5%.

IgG-Sepharose 4B bound BGP showed enhanced activity, 150% and 133% at 1.0% (w/v) and 2.0% (w/v) of CTAB, respectively whereas the soluble counterpart exhibited activation up to 119% and 104% of its initial activity respectively under identical conditions.

The immobilized enzyme retained 91% of its original activity at 1.0% (w/v) of the Surf Excel exposure whereas the soluble form of the enzyme exhibited only 62% activity under similar treatment. At 2.0% (w/v) Surf Excel, immobilized enzyme retained 48% activity, whereas the soluble BGP exhibited a loss of nearly 88% of its initial activity.

5.3.4. Kinetic Analysis

K_m obtained from Lineweaver Burk plots was 1.3 mM for both soluble and immobilized BGP preparations. The k_{cat} for soluble BGP was 2.7 s^{-1} while that for immobilized preparation was 2.0 s^{-1}.

5.4. DISCUSSION

The enzyme electrodes based on immobilized peroxidase have been successfully employed for measuring various types of chemical pollutants (Gasper *et al.*, 2000; Schumacher *et al.*, 2001; Wang *et al.*, 2001; Alpeeva *et al.*, 2005). The rare availability and high cost of the commercially purified HRP has limited its use for various reasons. In order to circumvent such problem, it is advantageous to use ammonium sulphate fractionated bitter gourd proteins instead of pure enzyme for the immobilization of peroxidase. Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure (Muller and Zwing, 1982). A large number of earlier studies have described that the enzymes immobilized on the antibody support showed very high effectiveness factor (Jan *et al.*, 2001; Jan and Husain, 2004). The specific binding of peroxidase to the immunoaffinity support directly from the ammonium sulphate precipitated proteins of bitter gourd is significantly useful in reducing the cost of immobilized enzyme preparation.

Immunoaffinity bound BGP was significantly more stable against urea and GdnCl denaturation and it suggested that such preparation could be exploited even in the presence of these chaotropic agents. Urea and GdnCl are very frequently used as strong denaturants of proteins, which have been shown to undergo pronounced structural changes at concentrations greater than 1.0 M of these denaturants (Tanford, 1968; Tanford, 1970). However, the action mechanism of urea induced denaturation of proteins structure has not been clearly known, several earlier studies have proposed that protein is unfolded by the direct interaction of urea molecule with a peptide backbone via hydrogen bonding/hydrophobic interaction, which contributes to the maintenance of protein conformation (Makhatadze and Privalor, 1992).

Immobilized BGP exhibited remarkably high stability against the water-miscible organic solvents induced inactivation. The significance of support matrices for the utilization of enzymes in organic solvents has already been recognized (Batra and Gupta, 1994; Kulshrestha and Husain, 2006) and it has been shown that proteinic supports may also be useful (Jan *et al.*, 2001; Akhtar *et al.*, 2005a., Khan *et al.*, 2005; Matto and Husain, 2006). More recently our group has shown that IgG-Co^{2+}-Sepharose/IgG-Sepharose bound glucose oxidase was significantly stable against the inactivation mediated by pH, urea, heat and water-miscible organic solvents (Jan *et al.*, 2001; Jan and Husain, 2004). The significance of other supports in the stabilization of peroxidases against water-miscible organic solvents has also been described (Kulshrestha and Husain, 2006).

Immunoaffinity bound BGP showed an enhancement in its activity even when it was exposed to a very high concentration of cationic detergent, CTAB while this activation was decreased in case of soluble BGP (Table 10). We have earlier shown that BGP immobilized on Con A-Sephadex support was also activated by the exposure of lower concentration of detergent (Akhtar *et al.*, 2005a). It has been shown by other workers that some enzymes exhibited enhanced activity in water/detergent media owing to the positive interactions between enzyme and detergent (Viparelli and Francesco, 1999). This bioaffinity bound BGP preparation was quite resistant to the inactivation induced by anionic detergent, SDS (Akhtar *et al.*, 2005a). In view of its high stability in the presence of detergents, it is possible to use such preparations for the treatment of wastewater contaminated with various organic pollutants along with detergents. Some earlier reports indicated that the peroxidases immobilized by other

methods also resulted into stabilization against the exposure caused by detergents (Kulshrestha and Husain, 2006). The K_m values determined for immobilized and soluble BGP were same while the k_{cat} for soluble BGP was slightly higher than immobilized preparation. Storage stability studies performed in our lab indicated that immobilized BGP was quite stable and retained 93% of activity even after two months of storage at 4 ^{o}C.

This study suggested that the enzyme bound to the IgG-Sepharose 4B support could be used more effectively for the conversion of various compounds, which are insoluble or sparingly soluble in aqueous environment in the presence of high concentrations of water-miscible organic solvents and detergents. The generally observed higher stability of the immunoaffinity bound BGP against various forms of inactivation may be related to the specific and strong binding of enzyme with antibody support which prevent the unfolding/denaturation of enzyme. In view of the improved stability of immunoaffinity bound BGP against heat, pH, chaotropic agents, detergents, proteolytic enzyme and water-miscible organic solvents, it may find a large number of applications especially in the construction of enzyme-based analytical devices for clinical, environmental and food technology (Gasper *et al.*, 2000; Alpeeva *et al.*, 2005).

The main objective of this work was to cut down the cost of the enzyme purification and to immobilize the enzyme at the step of purification, which can turn out to be of great interest in the area of clinical and environmental analysis. This immobilized preparation was remarkably stable against the inactivation mediated by heat, pH, chaotropic agents, detergents and water-miscible organic solvents. Such preparations could be easily employed in the analysis of water soluble and insoluble compounds, synthesis of novel organic compounds, solvent engineering and protein engineering.

Summary

Bitter gourd (*Momordica charantia*) is an important medicinal plant, which is easily available in most parts of India. Plant peroxidases are receiving increasing attention due to their extensive bioactivation property and potential applications in clinical, biochemical, biotechnological, and related areas.

Peroxidase from bitter gourd was purified by ammonium sulphate fractionation followed by gel filtration and affinity chromatography. The enzyme was purified to 42 fold with the retention of 67% of the initial activity. The enzyme exhibited its maximum activity at pH 5.6 and 40 °C. The enzyme retained half of its activity after 1 h incubation at 60 °C. Molecular weight of the purified glycosylated bitter gourd peroxidase determined by Sephacryl S-100 and SDS-PAGE was 43 kDa. Stokes radius, diffusion coefficient and sedimentation coefficient of the purified peroxidase were 27.3 Å, 8.17 x 10^{-7} cm^2/sec and 3.74 S, respectively. K_m for *o*-dianisidine and ABTS were 1.3 and 4.9 mM, respectively. The activity of the enzyme was inhibited by sulfide, azide and L-cysteine. The sulphydryl groups of the enzyme were 16 mmoles/mole of the protein. Bitter gourd peroxidase shares its properties with the typical class III peroxidases.

Glycosylation has been shown to play an important role in protein folding, biological activity, protein stability and immunogenicity. Majority of plant peroxidases have been reported to be glycosylated. The purified bitter gourd peroxidase obtained as a single band was found to be glycosylated whereas the two other isoenzymes obtained were non-glycosylated. The carbohydrate content of purified bitter gourd peroxidase was 25% (w/w) mass of the protein. Purified bitter gourd peroxidase gave a pink colored band when stained by periodic acid schiffs reagent.

The possible role of carbohydrate moieties in the stabilization of proteins was investigated by using bitter gourd peroxidase as a model system. A comparative study of glycosylated and non-glycosylated isoenzymes of bitter gourd peroxidase was performed at various temperatures, pH, water-miscible organic solvents, detergents and chaotropic agents; like urea. The pH and temperature-optima of both glycosylated and non-glycosylated isoforms of bitter gourd peroxidase remained unchanged. Glycosylated bitter gourd peroxidase retained more activity than non-glycosylated preparation at various temperatures, other than the temperature-optima. Glycosylated bitter gourd peroxidase was also significantly more stable than non-glycosylated

preparation when incubated at different time intervals at 60 °C. The glycosylated form of bitter gourd peroxidase retained a higher enzyme activity as compared to the non-glycosylated bitter gourd peroxidase when exposed to various detergents, SDS, Tween-20 and Triton X-100. Glycosylated bitter gourd peroxidase also retained greater fraction of catalytic activity when exposed to various water-miscible organic solvents like dimethyl sulphoxide and dimethyl formamide. Thus, glycosylated bitter gourd peroxidase retained significantly more catalytic activity against the exposure caused by various physical and chemical denaturants. Non-glycosylated bitter gourd peroxidase exhibited higher intrinsic fluorescence intensity as compared to glycosylated bitter gourd peroxidase due to greater exposure of fluorophore in this form. Unfolding of both forms of bitter gourd peroxidase in the presence of high urea concentrations, studied by fluorescence measurement indicated greater perturbations in the conformation of non-glycosylated preparation. The different properties examined thus indicated that glycosylation played an important role in the stabilization of native conformation of proteins against the inactivation caused by various types of denaturants.

Horseradish peroxidase has dominated the world of peroxidases since a long time. It is widely used in diagnostic, biosensing and biotechnological applications. However, the availability and high cost of commercially available horseradish peroxidase restricts its applications. The structural and functional aspects of bitter gourd peroxidase and horseradish peroxidase have been compared for their stability against the denaturation induced by heat, pH, urea, SDS, and water-miscible organic solvents. Stability of the enzymes was monitored spectrophotometrically as well as by ellipticity changes in far and near UV-CD region. Bitter gourd peroxidase was more thermo-stable as compared to horseradish peroxidase. Temperature activity profiles of bitter gourd peroxidase and horseradish peroxidase exhibited similar temperature-optima at 40 °C. Bitter gourd peroxidase retained a higher fraction of catalytic activity as compared to horseradish peroxidase when incubated at various temperatures (30-80 °C). The disruption of secondary and tertiary structure at various temperatures was greater for horseradish peroxidase. The secondary structure of bitter gourd peroxidase remained stable till 72 °C whereas horseradish peroxidase lost substantial secondary structure from 60-80 °C. This observation was further supported by far UV-CD spectra of bitter gourd peroxidase at 30 °C, 60 °C and 80 °C. Bitter gourd peroxidase

retained remarkably greater fraction of enzyme activity as compared to horseradish peroxidase in the alkaline pH range. The difference in catalytic activity of bitter gourd peroxidase by varying the pH was related to the change in secondary structure as manifested by the change in the CD value at 222 nm. It was further complemented by the far UV-CD spectra, which showed greater retention of secondary structure at pH 6.0 and pH 10.0. Bitter gourd peroxidase showed remarkable stability in the presence of urea. There was no change in the enzymatic activity of both bitter gourd peroxidase and horseradish peroxidase till 2.0 M urea exposure. However, there was a significant decrease in enzymatic activity beginning from 4.0 M urea. Bitter gourd peroxidase retained greater fraction of catalytic activity when exposed to 4.0 M urea for varying times. The retention of catalytic activity by bitter gourd peroxidase was remarkably greater than horseradish peroxidase when exposed to higher concentration of urea (8.0 M) for 2 h. Bitter gourd peroxidase and horseradish peroxidase exhibited an enhancement in enzyme activity when exposed to SDS. However, the enhancement in enzyme activity was more for bitter gourd peroxidase as compared to horseradish peroxidase. Both peroxidases were activated by the exposure to 20-60% (v/v) of dimethyl formamide. However, there was more activation in enzyme activity of bitter gourd peroxidase as compared to horseradish peroxidase activity. Bitter gourd peroxidase activity was markedly enhanced and it was more stable than horseradish peroxidase on exposure to 10-60% (v/v) dimethyl suphoxide. In view of its higher stability, bitter gourd peroxidase can serve as a better alternative to horseradish peroxidase in clinical, biochemical, analytical, and environmental analyses as well as in various biotechnological applications.

Purified bitter gourd peroxidase was injected into healthy male albino rabbits for the production of anti bitter gourd peroxidase polyclonal antibodies. Antibodies raised against bitter gourd peroxidase were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate fractionated antiserum was passed through DEAE-cellulose column. The fractions containing purified antiperoxidase antibodies were pooled for further use. DEAE-cellulose purified IgG loaded on SDS-PAGE clearly showed two bands corresponding to the heavy and light chains of the antibody. The position of migration of IgG subunits correspond to apparent molecular weights of 45 kDa and 20 kDa. The native PAGE showed a single band and this result supported the purity of the purified antibodies. Purified anti-bitter

gourd peroxidase antibodies gave a clear precipitin line with the purified bitter gourd peroxidase when Ouchterlony double immunodiffusion was performed. A high titer was obtained through direct binding enzyme linked immunosorbent assay.

The IgG isolated were used for the construction of Sepharose 4B-anti bitter gourd peroxidase immunoaffinity support. Polyclonal antibody bound Sepharose 4B support was exploited for the immobilization of bitter gourd peroxidase directly from ammonium sulphate precipitated proteins. Immunoaffinity immobilized bitter gourd peroxidase exhibited high yield of immobilization. The immunoaffinity immobilized bitter gourd peroxidase preparation exhibited very high effectiveness factor (η) value of 0.96. Immobilized enzyme exhibited no change in temperature-optima between 30-40 °C whereas soluble bitter gourd peroxidase had temperature-optima at 40 °C. Immunoaffinity bound bitter gourd peroxidase retained greater fraction of enzyme activity on both sides of temperature-optima compared to its soluble counter part. IgG-Sepharose 4B bound bitter gourd peroxidase showed a higher stability against heat, pH, chaotropic agents (urea and guanidinium chloride), detergents (Cetyl trimethyl ammonium bromide and Surf Excel), proteolytic enzyme (trypsin) and water-miscible organic solvents; propanol, tetrahydrofuran and dioxane. The activity of immobilized bitter gourd peroxidase was significantly enhanced in the presence of cetyl trimethyl ammonium bromide and after treatment with trypsin as compared to soluble enzyme.

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LIST OF PUBLICATIONS AND PRESENTATIONS

List of publications:

1. Fatima, A., Husain, Q. Polyclonal antibodies mediated immobilization of a peroxidase from ammonium sulphate fractionated bitter gourd (*Momordica charantia*) proteins. *Biomol. Eng.* 2007 (in press).

2. Fatima, A., Husain, Q. A role of glycosyl moieties in the stabilization of bitter gourd (*Momordica charantia*) peroxidase. *Int. J. Biol. Macromolecules* 2007; **41:** 56-63.

3. Fatima, A., Husain, Q. Purification and characterization of a peroxidase from bitter gourd (Momordica charantia). *Protein and Peptide Lett.* 2007 (in press).

4. Fatima, A., Husain, Q. Novel peroxidase from bitter gourd (*Momordica charantia*) with enhanced stability against organic solvents and detergents: A comparison with horseradish peroxidase. *J. Mol. Cat.* B: Enzymatic 2007 (in press).

Seminars and symposia attended:

1. DBT sponsored workshop on "*Bioinformatics in the study of Biomacomolecules*"
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3. Polyclonal antibodies mediated simultaneous purification and immobilization of a novel peroxidase from bitter gourd (*Momordica charantia*).
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A role of glycosyl moieties in the stabilization of bitter gourd (*Momordica charantia*) peroxidase

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Abstract

The possible role of carbohydrate moieties in the stabilization of proteins has been investigated by using bitter gourd peroxidase as a model system. A comparative study of glycosylated and non-glycosylated isoenzymes of bitter gourd peroxidase was performed at various temperatures, pH, water-miscible organic solvents, detergents and chaotropic agent like urea. The pH-optima and temperature-optima of both glycosylated and non-glycosylated isoforms of bitter gourd peroxidase remained unchanged. The probes employed were changes in the enzyme activity and fluorescence. The glycosylated form of peroxidase retained greater fraction of enzyme activity against the exposure caused by various physical and chemical denaturants. The unfolding of both forms of enzyme in the presence of high urea concentrations, studied by fluorescence, indicated greater perturbations in the conformation of non-glycosylated preparation. The different properties examined thus indicated that glycosylation plays an important role in the stabilization of native conformation of proteins against the inactivation caused by various types of denaturants.

Keywords: Bitter gourd peroxidase; *Momordica charantia*; Glycosylation; Intrinsic fluorescence; Circular dichroism; Urea; Stabilization; Isoenzymes

1. Introduction

It is now well documented that glycosylation plays an important role in the stabilization of proteins. It has become an interesting field of research for many years. The covalent attachment of carbohydrate to proteins is a very common co- or post-translational event in the biosynthesis of glycoproteins, affecting a range of their physico-chemical and biological properties [1]. The period of proteomics has followed the era of genomics bringing back the study of glycans into focus [2]. A number of questions related to the specific and non-specific functions of glycoproteins, their structure and their wider implications in health and disease are still being explored [3]. They have attracted attention from medicine, for example, there have been studies on the treatment of *N*-linked glycoproteins of HIV with plant lectins [4]. There have been studies on the role of glycosylation in rheumatoid arthritis and malignant alteration of *N*-glycosylation in various tumors enabling glycobiology to expand to diverse fields such as glycopathology [5]. Glycoproteins have assumed biotechnological significance due to their immense therapeutic potential [6]. Plants have the ability to produce glycosylated proteins, which can be administered as oral vaccines [7]. Glycosylated proteins also play an important role in mediating 'specific recognition' events and a number of biological processes [8]. In mammals, glycosylation has been shown to be involved in protein folding, biological activity, protein stability and immunogenicity [9]. Glycosylated residues in plants could have similar functions or an extension of these roles [10]. Plant glycoproteins seem to carry the same limited set of structures regardless of the species [11]. Asparagine *N*-linked glycans are commonly found in plant glycoproteins [12]. It has earlier been reported that plant *N*-glycans carry a fucose residue in α1,3-linkage to the innermost GlcNAc and a xylose residue is also present [13]. Even though protein glycosylation is so abundant in nature, a lot still remains to be explored in terms of how the carbohydrate attachments affect peptide and protein activity [14].

A number of studies have been performed on glycosylated and deglycosylated/non-glycosylated proteins. Among plant

Abbreviations: BGP, bitter gourd peroxidase; far UV-CD, far ultra violet-circular dichroism; DMSO, dimethyl sulphoxide; DMF, dimethyl formamide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Fi, intrinsic fluorescence; GlcNAc, *N*-acetylglucosamine; PAS, periodic acid schiff

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proteins, most peroxidases have been reported to be glycosylated and are an ideal model to further explore the significance of glycosylated residues in relation to proteins [15]. A number of peroxidases, such as horseradish peroxidase, soybean peroxidase and lupin extensin peroxidase are glycosylated [16]. Cationic peanut peroxidase has also been shown to be glycosylated [3]. Three forms of anionic peroxidase purified from cucumber cotyledons have been shown to be glycosylated [12]. More recently, all three of the peroxidase isoenzymes purified from broccoli stems have also been shown to be glycosylated [17].

In the present study, a comparative investigation of enzymatic activity and structural stability of glycosylated and non-glycosylated isoforms of bitter gourd peroxidase (BGP) was carried out over a wide range of temperature, pH, detergents and organic solvents. The stability profiles of the two forms of BGP exposed to high urea concentrations was also studied in detail by employing fluorescence. This paper is a modest attempt to highlight the stabilizing role of glycans for plant proteins in general and plant peroxidases in particular.

2. Materials and methods

2.1. Materials

Sephacryl S-100, methyl α-D-mannopyranoside and reagents for electrophoresis were obtained from Sigma Chem. Co, (St. Louis, MO) USA. *o*-dianisidine-HCl was the product of IGIB, New Delhi, India. Hydrogen peroxide was obtained from Merck, India. Ammonium sulphate, urea, dimethyl sulphoxide, dimethyl formamide and sodium dodecyl sulphate, Triton X-100, Tween-20 were purchased from SRL Chemicals, Mumbai, India. Concanavalin A (Con A)-Sepharose was obtained from Genei Chemicals, Bangalore, India. Bitter gourd was obtained from the local market. All the other chemicals and reagents used were of analytical grade.

2.2. Purification of glycosylated and non-glycosylated BGP

Bitter gourd proteins were precipitated by ammonium sulphate fractionation [18]. The salt fractionated and dialyzed BGP was filtered through whatman filter paper. The enzyme was then concentrated. The Sephacryl S-100 column (49 cm × 1.7 cm) was equilibrated with 100 mM sodium acetate buffer, pH 5.6. The dialyzed, filtered and concentrated BGP was then loaded on the column. Fractions of 2.0 ml were collected using 100 mM sodium acetate buffer, pH 5.6. Protein concentration and peroxidase activity were determined in all collected fractions. Con A-Sepharose column was equilibrated with 100 mM sodium acetate buffer, pH 5.6 containing 1.0 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.15 M NaCl. The fractions obtained from the main peak of Sephacryl S-100 column were then pooled and passed through the bioaffinity column. The unbound BGP obtained in the washing buffer represented the non-glycosylated preparation. Bioaffinity adsorbed proteins were eluted by passing 100 mM sodium acetate buffer, pH 5.6 containing 0.5 M methyl α-D-mannopyranoside. The eluted protein represented glycosylated BGP [19].

2.3. Measurement of peroxidase activity

Peroxidase activity was determined from the change in the optical density at (λ_{460} nm) by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine-HCl in the presence of 18 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.6, for 15 min at 37 °C [18].

One unit of peroxidase activity is defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 μmol of *o*-dianisidine-HCl per minute at 37 °C into colored product (εm at 460 = 30,000 M^{-1} cm^{-1}).

2.4. Protein assay

Protein concentration was determined using the procedure described by Bradford [20]. Bovine serum albumin was used as a standard protein.

2.5. Polyacrylamide gel electrophoresis

All electrophoreses were performed by the procedure described by Laemmli [21]. Electrophoresis was carried out in vertical slab gels, which were run at a constant voltage of 50 V. The gels (10%) were pre-run for 1 h before loading the samples. Stacking gels were used with a difference in the concentration of acrylamide in upper and lower gels. Native PAGE (10%) was run and substrate staining was performed using 18 mM H_2O_2 and 6 mM *o*-dianisidine-HCl. SDS-PAGE was also run and the gel was stained for visualization by using silver nitrate solution [22]. A SDS-PAGE (10%) was also run and stained for glycoprotein by using PAS reagent [23].

2.6. Effect of temperature

Activity of glycosylated and non-glycosylated preparations of BGP (0.4 U/ml) was determined at various temperatures (30–80 °C) in 100 mM sodium acetate buffer, pH 5.6. The percent remaining enzyme activity was calculated by taking activity at temperature-optimum as control (100%).

In another set of experiment, glycosylated and non-glycosylated BGP preparations were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.6. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and then the peroxidase activity was determined as described in the text. The percent remaining activity was determined by considering the activity of enzyme unincubated at 60 °C as control (100%).

2.7. Effect of pH

An appropriate and equal amount of glycosylated and non-glycosylated BGP preparations were added for determining the activity of enzyme in the buffer of different pH values. The buffers used were glycine–HCl buffer (2.0 and 3.0); sodium acetate buffer (4.0–6.0); sodium phosphate buffer (7.0 and 8.0); Tris–HCl buffer (9.0 and 10.0). The remaining percent activ-

ity was calculated by taking activity at pH-optimum as control (100%).

2.8. Effect of urea

Glycosylated and non-glycosylated BGP (0.4 U/ml) were incubated with increasing concentrations of urea (2.0–8.0 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 °C.

In another set of experiment, both the preparations of BGP (0.4 U/ml) were incubated with 4.0 M urea for varying time intervals. Peroxidase activity was determined after each incubation period. The activity of the urea untreated enzyme was considered as control (100%) for calculating the remaining percent activity.

2.9. Effect of organic solvents

Glycosylated and non-glycosylated BGP (0.4 U/ml) were incubated with varying concentrations of water-miscible organic solvents, DMSO and DMF (10–60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of enzyme without organic solvent was taken as control (100%) for calculating the remaining percent activity.

2.10. Effect of detergents

Glycosylated and non-glycosylated BGP (0.4 U/ml) were incubated with varying concentrations of detergents: SDS (0.1–1.0%, w/v), Tween-20 (0.5–5.0%, v/v), Triton X-100 (0.5–5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of the enzyme without detergent was considered as control (100%) for calculation of percent remaining activity.

2.11. Denaturant induced studies (fluorescence measurements)

Fluorescence measurements were performed on a Shimadzu Spectrofluorometer, Model RF-540. The protein concentration was taken to optimize the measuring conditions. Sample solutions containing appropriate concentration of BGP were incubated with varying concentrations of urea (0–8.0 M) at 30 °C for 6 h. The intrinsic fluorescence was taken by exciting the protein at 280 nm and the fluorescence emission spectra was recorded between 300 and 400 nm for each denaturant concentration.

The average emission wavelength ($\lambda_{av.em}$) was calculated according to the equation

$$\lambda_{av.em} = \frac{\sum(Fi\,\lambda_i)}{\sum Fi}$$

where Fi is the fluorescence intensity and λ is the wavelength, this parameter reflects changes in the shape of the spectrum as well as in position [24].

2.12. Statistical analysis

The data expressed in various studies was plotted using Sigma Plot-5 and expressed as S.E. (±).

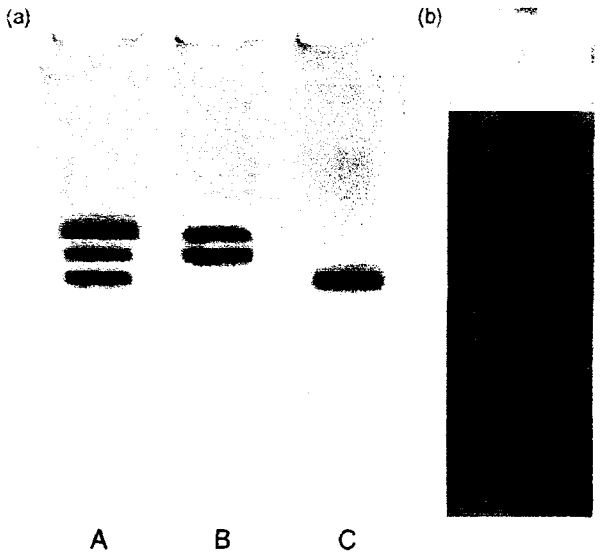

Fig. 1. (a) BGP proteins from various steps, activity stained on 10% native PAGE. Pooled fractions of Sephacryl S-100 (Lane A), unbound proteins from Con A-Sepharose column, non-glycosylated preparation (Lane B), Con A-Sepharose bound and then eluted proteins, glycosylated BGP (Lane C). Activity staining was performed by using 18 mM H_2O_2 and 6.0 mM *o*-dianisidine-HCl. (b) PAS staining of glycosylated BGP. A SDS PAGE (10%) was run. The peroxidase was stained by PAS reagent using the procedure described in Section 2.

3. Results

3.1. Glycosylated and non-glycosylated BGP

Sephacryl S-100 column was employed for purification of ammonium sulfate fractionated bitter gourd proteins, the pooled fractions of the activity and protein peaks were subjected to a stacking native PAGE (10%) and three, closely placed isoforms of BGP were obtained on substrate staining (Fig. 1a, lane A). The unbound BGP obtained in the washing revealed the presence of two non-glycosylated bands of BGP on substrate staining (Fig. 1a, lane B). The bound glycosylated proteins eluted from the bioaffinity support showed a single band when stained with the substrate *o*-dianisidine-HCl on the native gel (Fig. 1a, lane C). This eluted protein also gave a single band on SDS-PAGE when stained with silver nitrate (data not given). Glycosylated BGP gave a pink colored band at the region of enzyme activity when the gel was stained with PAS reagent on native PAGE (Fig. 1b). The molecular weight of glycosylated BGP was 43 kDa. The specific activity of glycosylated BGP was 8769 while that of the two non-glycosylated forms of BGP was 5158. The fold purification of BGP was 42 while that of non-glycosylated BGP was 25.

3.2. Effect of temperature

Both forms of BGP showed same temperature-optima, 40 °C. However, glycosylated BGP retained significantly more activity than non-glycosylated preparation at temperatures other than temperature-optima. Glycosylated BGP showed greater fraction of catalytic activity (80%) at 60 °C while the non-glycosylated

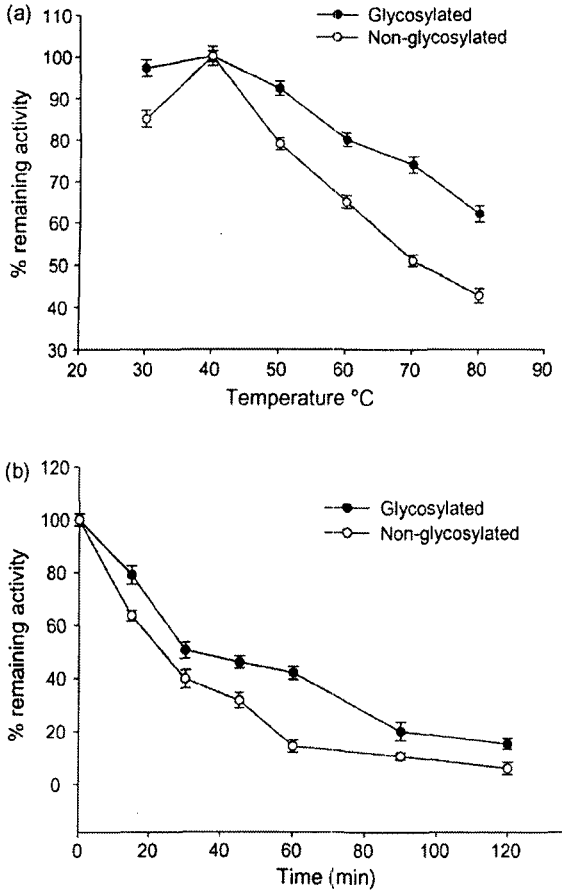

Fig. 2. (a) Temperature–activity profiles for glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated BGP preparations (0.4 U/ml) were assayed at various temperatures (30–80 °C) in 100 mM sodium acetate buffer, pH 5.6. Activity expressed at 40 °C was taken as control (100%) for calculating percent activity. (b) Thermal denaturation of glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated preparation of BGP (0.4 U/ml) were incubated at 60 °C for varying times in 100 mM sodium acetate buffer, pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined as described in the text. Un-incubated samples at 60 °C were taken as control (100%) for the calculation of percent activity.

form retained only 65% activity. Moreover, glycosylated BGP retained 74% activity at 70 °C whereas non-glycosylated preparation lost nearly 50% activity at the same temperature (Fig. 2a).

Glycosylated BGP was significantly more stable than non-glycosylated preparation when incubated for different time intervals at 60 °C. The former retained 42% activity after 1 h of incubation at 60 °C whereas the latter exhibited marginal activity of 14% under similar incubation conditions (Fig. 2b).

3.3. Effect of pH

There was not much difference in the pH–activity profiles of glycosylated and non-glycosylated BGP (Fig. 3). Both forms exhibited similar pH optima, pH 6.0. There was no detectable difference in far-UV CD spectra of the two forms at various pH values indicating no change in secondary structures of both forms of BGP (data not shown).

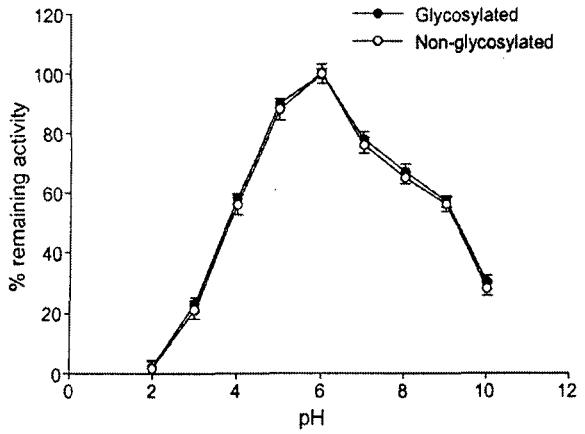

Fig. 3. pH–activity profiles of glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated preparations of BGP (0.4 U/ml) were incubated in buffers of different pH values. The buffers used were glycine HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0–6.0), sodium phosphate (pH 7.0 and 8.0) and Tris–HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Activity expressed at pH 6.0 was taken as control (100%) for calculating percent remaining activity.

3.4. Effect of urea

The inactivation of both forms of BGP at different concentrations of urea has been demonstrated in Fig. 4a. There was no significant change in the activity of both forms of BGP after their incubation with 2.0 M urea for 2 h. However, the change in catalytic activity became more pronounced from 4.0 M urea concentration onwards. Glycosylated form retained a remarkably very high activity, 90% after exposure to 6.0 M urea for 2 h whereas non-glycosylated forms lost nearly 68% of the original activity under similar incubation conditions. Further incubation of both types of BGP with 8.0 M urea for 2 h resulted in a loss of 71% activity for non-glycosylated preparation whereas glycosylated BGP retained 88% of the initial activity.

Fig. 4b shows the urea-induced inactivation of glycosylated and non-glycosylated BGP. Glycosylated BGP was remarkably more resistant to inactivation induced by 4.0 M urea as compared to non-glycosylated BGP. Glycosylated BGP retained 90% activity even after 2 h of incubation with 4.0 M urea while the non-glycosylated preparation showed 40% of the initial activity under identical treatment.

3.5. Effect of organic solvents

The effect of increasing concentration of water-miscible organic solvents; DMSO and DMF, (10–60%, v/v) on the activity of glycosylated and non-glycosylated BGP is shown in Table 1. There is a conspicuous activation in both glycosylated and non-glycosylated preparations of BGP when treated with water-miscible organic solvents. The activity of glycosylated BGP enhanced to 210% as compared to non-glycosylated preparation which exhibited an enhancement up to only 132% when treated with 30% (v/v) DMSO. At 60% (v/v) DMSO, glycosylated BGP activity was increased to 110% whereas non-glycosylated preparation showed no activation. Exposure to 40% (v/v) DMF resulted in an enhancement of 140% of glycosy-

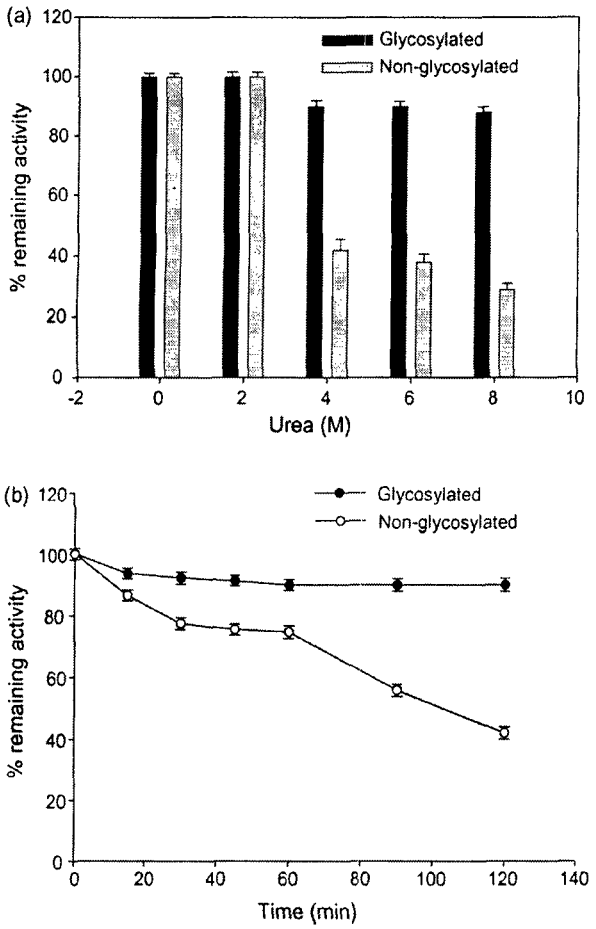

Fig. 4. (a) Effect of increasing concentration of urea on glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated BGP preparations (0.4 U/ml) were incubated in 2.0–8.0 M urea in 100 mM, sodium acetate buffer, pH 5.6 at 37 °C for 2 h. Enzyme activity was determined as mentioned in the text. For calculating the percent activity untreated samples were considered as control (100%). (b) Effect of 4.0 M urea on glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated BGP preparations (0.4 U/ml) were incubated in 4.0 M urea in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for varying times. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the percent activity urea untreated samples were considered as control (100%).

lated BGP activity while the non-glycosylated BGP exhibited a slight enhancement in enzyme activity. The activity of glycosylated and non-glycosylated BGP was enhanced to 142 and 106%, respectively at 60% (v/v) DMF exposure.

3.6. Effect of detergents

The effect of increasing concentration of SDS (0.1–1.0%, w/v) on the activity of both types of BGP has been shown in Fig. 5a. The activity of glycosylated BGP was enhanced significantly to 231% by the exposure of 1.0% (w/v) SDS whereas the non-glycosylated preparation was activated to 112% in the presence of 0.4% (w/v) SDS. The activity of glycosylated form was enhanced markedly to a very high value, 786% at 0.3% (w/v) SDS whereas the non-glycosylated preparation was activated to less than half of this value at the same concentration of SDS.

Pre-incubation of both types of BGP with Tween-20 (0.5–5.0%, v/v) at 37 °C for 1 h resulted in a greater loss of activity for non-glycosylated forms than the glycosylated form (Fig. 5b). Glycosylated form retained more than 50% activity at a concentration of 2.5% (v/v) Tween-20 whereas the non-glycosylated preparation lost nearly 62% activity under similar exposure. At a concentration of 5.0% (v/v) Tween-20, glycosylated form still showed 44% peroxidase activity while the non-glycosylated forms retained a marginal activity of 21% under the similar incubation conditions.

The effect of increasing concentration of Triton-X 100 (0.5–5.0%, v/v) is depicted in Fig. 5c. The peroxidase activity of glycosylated BGP was enhanced till a concentration of 1.5% (v/v) reaching a high of 146% at 0.5% (v/v) Triton-X 100 and reaching a closer value of 143% activity at 1% (v/v) Triton X-100. The non-glycosylated preparation was activated only till a concentration of 0.5% (v/v) where it retained 117% peroxidase activity. Glycosylated form retained a higher enzyme activity as compared to the non-glycosylated forms, for example it retained 84% activity at 5.0% (v/v) Triton X-100 whereas the latter had only 63% activity under the identical incubation conditions. There was a leveling off in the activity of both glycosylated and non-glycosylated preparation of BGP from 3% (v/v) Triton X-100. The intrinsic fluorescence of glyco-

Table 1
Effect of DMF and DMSO on glycosylated and non-glycosylated BGP

Organic solvent (%, v/v)	Percent remaining activity			
	DMF		DMSO	
	Glycosylated	Non-glycosylated	Glycosylated	Non-glycosylated
0	100	100	100	100
10	92 ± 2.30	85 ± 1.67	121 ± 1.79	109 ± 1.81
20	130 ± 2.00	126 ± 2.24	203 ± 1.91	149 ± 1.88
30	137 ± 1.76	127 ± 1.63	209 ± 2.09	132 ± 1.99
40	142 ± 3.30	112 ± 2.50	174 ± 3.00	125 ± 2.79
50	142 ± 2.80	106 ± 3.00	146 ± 1.77	113 ± 1.52
60	142 ± 2.05	106 ± 1.82	110 ± 2.33	96 ± 2.12

Glycosylated and non-glycosylated BGP (0.4 U/ml) were incubated with increasing concentration of DMSO and DMF (10–60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. The values are mean ± S.D. of three independent experiments performed in duplicate.

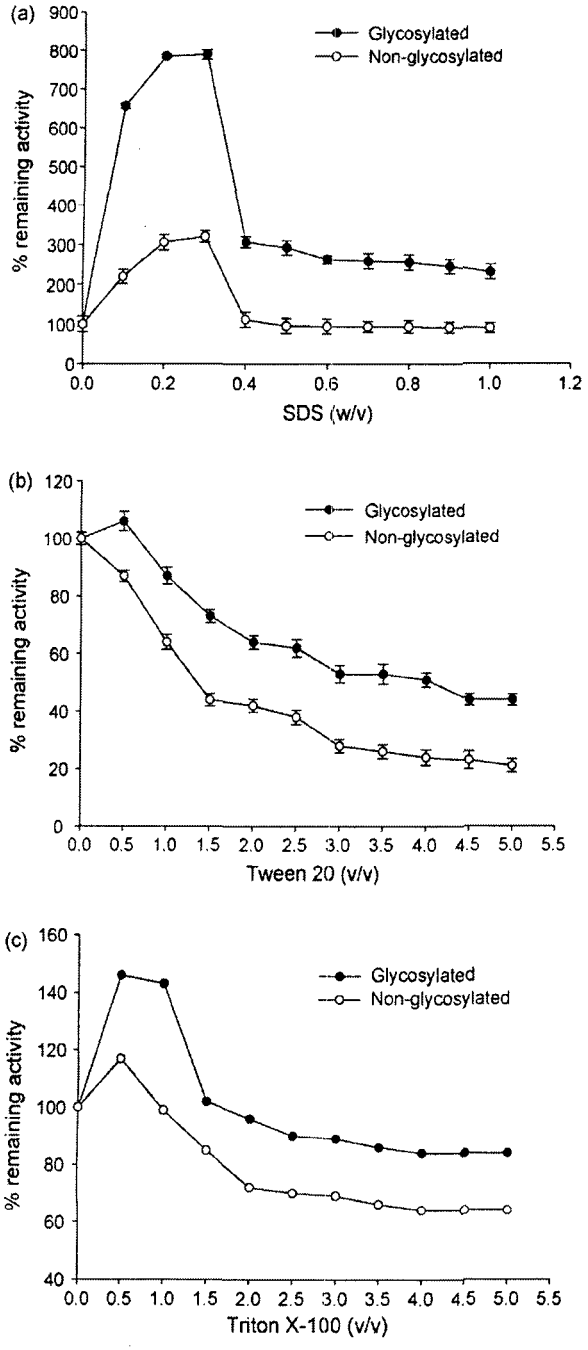

Fig. 5. (a) Effect of SDS on glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated preparations of BGP (0.4 U/ml) were incubated with increasing concentration of SDS (0.1–1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. SDS unexposed samples activity was taken as control (100%) for calculating percent remaining activity. (b) Effect of Tween-20 on glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated BGP preparations (0.4 U/ml) were incubated with increasing concentration of Tween-20 (0.5–5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. Tween 20 untreated samples were taken as control (100%) for calculating percent remaining activity. (c) Effect of Triton X-100 on glycosylated and non-glycosylated BGP. Glycosylated and non-glycosylated BGP preparations (0.4 U/ml) were incubated with increasing concentration of Triton X-100 (0.5–5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. Triton X-100 untreated samples were taken as control (100%) for calculating percent remaining activity.

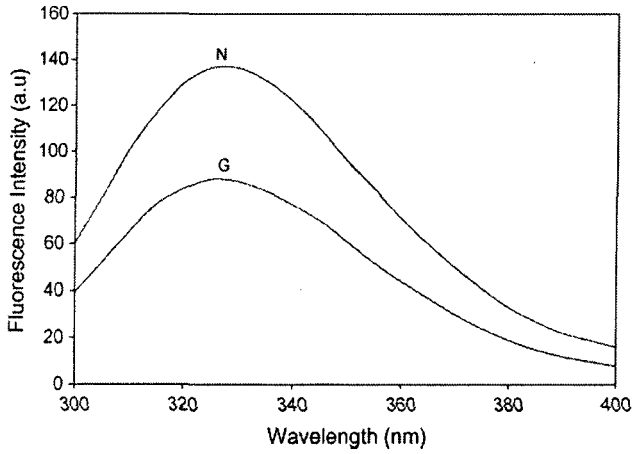

Fig. 6. Intrinsic fluorescence of glycosylated and non-glycosylated BGP. The solutions of glycosylated and non-glycosylated BGP preparations were prepared in 100 mM sodium acetate buffer, pH 5.6 and their intrinsic fluorescence was performed on Shimadzu Spectrofluorometer, Model RF-1501. The protein concentration was chosen to stabilize the measuring conditions. The excitation wavelength was 280 nm and the emission wavelength was taken between 300–400 nm. The G represents glycosylated BGP and the N represents non-glycosylated BGP preparation.

sylated and non-glycosylated preparation has been shown in Fig. 6.

3.7. Fluorescence studies of urea treated BGP

Fig. 7a demonstrates the unfolding pathway of glycosylated and non-glycosylated BGP in the presence of high urea concentrations (0–8.0 M). There was an increase in fluorescence intensity (Fi) when compared to Fi of control from 1.0 to 2.0 M urea for both the forms of BGP. The maximum increase in Fi was at 1.0 M urea. The non-glycosylated preparation showed a higher Fi (205 a.u.) at 1 M urea as compared to the glycosylated form (136 a.u.). The glycosylated form exhibited a leveling off of Fi from 5.0 to 7.0 M urea, which was followed by a decline in the Fi at 8.0 M urea. This constant phase was absent in the case of non-glycosylated preparation, which showed a decline in Fi from 136 a.u. at 5.0 M urea to 99 a.u. at 6.0 M urea. Glycosylated and non-glycosylated preparation had an almost identical Fi of 20 and 24 a.u. at 8.0 M urea, respectively. At each of the urea concentrations (0–7.0 M), the non-glycosylated preparation exhibited a higher Fi as compared to the glycosylated form.

The fluorescence $\lambda_{av.em}$ was plotted and analyzed as a function of high urea concentration (Fig. 7b). There was only a slight change (1–2 nm) in the $\lambda_{av.em}$ as compared to control in the case of both glycosylated and non-glycosylated BGP from 1 to 3.0 M urea. However, in both the forms the change was more pronounced from 4.0 to 8.0 M urea (3–12 nm). The $\lambda_{av.em}$ was slightly higher for non-glycosylated preparation than glycosylated form at each urea concentration.

The ratio of Fi at 306 and 350 nm ($F_{306/350}$) using an excitation wavelength of 280 nm can be regarded as contribution of tyrosine residues to fluorescence measurements [25]. A decrease in the $F_{306/350}$ is an indication of a red shift while an increase in the ratio is a signal of blue shift in the environment of tyro-

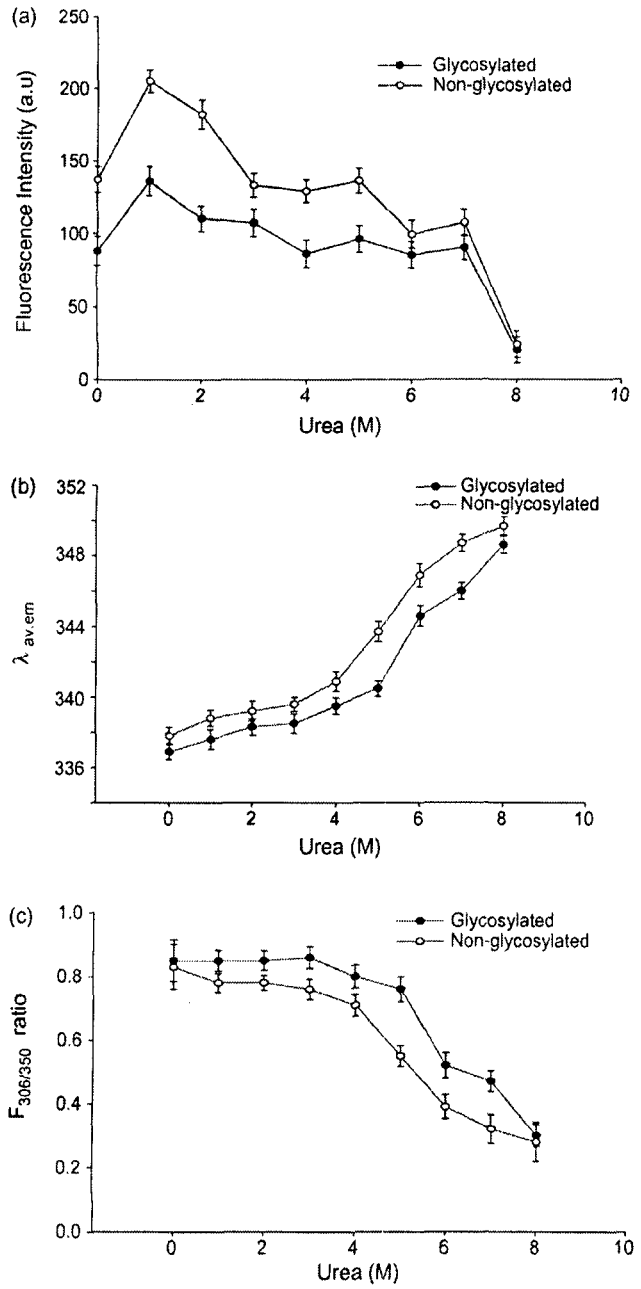

Fig. 7. (a) Effect of high urea concentration on fluorescence intensity of glycosylated and non-glycosylated BGP. The fluorescence intensity (a.u.) at high urea concentration (0–8.0 M) was measured for glycosylated and non-glycosylated BGP. The protein concentration was chosen to optimize the measuring conditions. The protein was excited at 280 nm. The preparations without urea were taken as control. (b) The average wavelength emission ($\lambda_{av.em}$) for glycosylated and non-glycosylated BGP at high urea concentrations. The average wavelength emission ($\lambda_{av.em}$) of 0–8.0 M urea treated glycosylated and non-glycosylated BGP preparations was measured. The protein concentration was chosen to optimize the measuring conditions. The proteins were excited at 280 nm. The preparations without urea were taken as control. (c) The ($F_{306/350}$) ratio of glycosylated and non-glycosylated BGP at high urea concentration. The ($F_{306/350}$) ratio of 0–8.0 M urea treated glycosylated and non-glycosylated BGP preparations was measured. The protein concentration was chosen to optimize the measuring conditions. The proteins were excited at 280 nm. The preparations without urea were taken as control.

sine residues. Fig. 7c demonstrates a plot of $F_{306/350}$ and urea concentration (0–8.0 M). No significant change in the $F_{306/350}$ was observed from 1.0 to 3.0 M urea (0.85) in the case of glycosylated BGP while the ratio was significantly decreased from 0.83 at 1.0 M urea to 0.71 at 4.0 M urea in the case of non-glycosylated preparation. At 5.0 M urea the ratio decrease in the case of non-glycosylated BGP (0.55) was more as compared to glycosylated form (0.76). At 8.0 M urea the ratio was nearly same as glycosylated form (0.3).

4. Discussion

Glycosylation is one of the most naturally occurring modifications of the covalent structure of proteins [26]. The effect of glycosylation on enzyme structure and activity has been studied by various workers [27]. Several roles have been suggested for the carbohydrate moieties of proteins one of which includes stabilization of protein conformation [28]. The present study evaluates the role of carbohydrate moieties in the stability of BGP. Glycosylated BGP was more stable than the non-glycosylated preparation over a wide range of temperatures studied (Fig. 2a). Both forms of BGP exhibited the same temperature-optima. The form devoid of carbohydrate residues has more flexibility resulting in diminished stability at higher temperatures [26]. The absence of glycans has a profound influence on thermo stability of proteins [29]. It has also been reported by earlier workers that glycosylation of various enzymes resulted in their thermal stabilization [30,31]. The thermal denaturation of both forms of BGP at 60 °C for various time intervals also showed greater retention of enzyme activity for glycosylated BGP (Fig. 2b). Carbohydrate moieties thus clearly indicate a role in thermal denaturation of enzyme [32]. There was not much difference in the pH–activity profiles of glycosylated and non-glycosylated BGP (Fig. 3). Both types of BGP exhibited similar pH-optima. There was no detectable difference in the far-UV CD spectra of the two forms at various pH values indicating no change in secondary structures of both forms of BGP at various pH values (data not shown).

Glycosylated BGP retained greater enzyme activity as compared to the non-glycosylated form when treated with different concentrations of urea and when exposed to 4 M urea at different time intervals (Fig. 4a and b, respectively). Some earlier workers have already shown that the presence of carbohydrate residues in proteins caused resistance to inactivation mediated urea [33]. In a recent study, Li et al. [34] has reported that a peroxidase from *Aedes aegypti* chorion is extremely resistant to inactivation induced by various denaturing agents [34]. The carbohydrate part of the protein thus contributes towards stabilization of protein against denaturants [35]. The percent remaining activity for glycosylated form was more as compared to the non-glycosylated preparation when exposed to various concentrations of detergents; SDS, Tween-20, Triton X-100 (Fig. 5a–c) and organic solvents; DMSO, DMF (Table 1). The carbohydrate moieties play an important role in the stabilization of enzymatic activity of BGP. It has already been described that turnip peroxidases require carbohydrate moieties for its stability [36].

Earlier workers have also shown that the activity of some enzymes was enhanced in water/detergent media, owing to the positive interactions between enzyme and detergent [37]. There was a three-fold increase in the activity of soybean peroxidase when the enzyme was treated with 0.1% (w/v) of SDS and Tween 20 [38].

It was found that the non-glycosylated form exhibited higher Fi due to greater exposure of fluorophore in this form (Fig. 6). Earlier workers have reported a similar increase in intrinsic fluorescence of the enzyme devoid of carbohydrate moiety [26]. The unfolding of the two forms in the presence of higher urea concentration (0–8.0 M) is demonstrated in Fig. 7a. A close to native like form was evident at 4.0 and 6.0 M urea for glycosylated BGP and at 3.0–4.0 M urea in the case of non-glycosylated BGP. At higher urea concentration Fi changed and the ($F_{306/350}$) ratio decreased (Fig. 7c) with a change in the $\lambda_{av.em}$ (Fig. 7b) for both the forms. This suggested a red shift with the tyrosine residues getting exposed to a more polar microenvironment. However, the change in Fi and ($F_{306/350}$) ratio was more in case of non-glycosylated preparation indicating that possibly the glycosylated form was shielded due to steric hindrance of carbohydrate residues. At 8.0 M urea both the forms were in a similar microenvironment demonstrated by an almost similar ($F_{306/350}$) ratio. Thus, there were overall changes in tertiary structure of both forms and a reorientation of tyrosine residues, which was more in case of non-glycosylated form.

The overall studies in this paper pointed towards an important role assumed by carbohydrate moieties in stabilizing a protein. Some earlier workers have also shown this by comparing the glycosylated and non-glycosylated forms of a protein [39]. The stabilization of peroxidases, in particular, from other sources due to presence of carbohydrates has also been reported [40].

Acknowledgements

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Polyclonal antibodies mediated immobilization of a peroxidase from ammonium sulphate fractionated bitter gourd (*Momordica charantia*) proteins

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Abstract

Polyclonal antibody bound Sepharose 4B support has been exploited for the immobilization of bitter gourd peroxidase directly from ammonium sulphate precipitated proteins. Immunoaffinity immobilized bitter gourd peroxidase exhibited high yield of immobilization. IgG-Sepharose 4B bound bitter gourd peroxidase showed a higher stability against heat, chaotropic agents (urea and guanidinium chloride), detergents (cetyl trimethyl ammonium bromide and Surf Excel), proteolytic enzyme (trypsin) and water-miscible organic solvents (propanol, THF and dioxane). The activity of immobilized bitter gourd peroxidase was significantly enhanced in the presence of cetyl trimethyl ammonium bromide and after treatment with trypsin as compared to soluble enzyme.

Keywords: Peroxidase; Immobilization; Stabilization; Organic solvents; Detergents; Polyclonal antibodies; Immunoaffinity support; Sepharose 4B

1. Introduction

Analytical technology based on enzymic biosensors is an extremely broad field, which has an impact on many industrial sectors such as the pharmaceutical, healthcare, food and agriculture industries as well as environmental monitoring (Alpeeva et al., 2005; Andreescu and Marty, 2006; Murphy, 2006). Enzyme immobilization on sensing electrode surfaces is one of the most important points to be considered in biosensor design. The selected procedure of immobilizing enzyme should be able to stabilize the macromolecules and allow easier diffusion of substrates and products to ensure an efficient electron transfer (Ghindilis et al., 1997). However, among immobilization methods being employed; very few can control the spatial distribution of catalyst. Stabilization of enzymes against the inactivation induced by numerous types of denaturants has been accomplished using a multitude of immobilization strategies including covalent coupling, adsorption, microencapsulation, polymer entrapment, chemical aggregation and bioaffinity, etc. (Husain and Jan, 2000; Duran and Esposito, 2000; Duran et al., 2002).

Procedures, which utilize the affinities of biomolecules and ligands for the immobilization of enzymes are gaining wider acceptance in the construction of sensitive enzyme-based analytical devices as well for other applications. The strong affinity of polyclonal/monoclonal antibodies for specific enzymes and those of lectin for glycoenzymes bearing appropriate oligosaccharides have been generally employed for this purpose (Saleemuddin and Husain, 1991; Saleemuddin, 1999; Bucur et al., 2005). Potential of affinity pairs like cellulose–cellulose binding domain bearing enzymes and immobilized metal ion-surface histidine bearing enzymes has also been recognized (Saleemuddin, 1999). Bioaffinity-based methods have several advantages over the other known methods used for the immobilization of enzymes. These procedures in view of their reversibility, lack of chemical modification and the usually accompanying stability enhancement are emerging as powerful tools for the immobilization of enzymes (Saleemuddin,

Abbreviations: BGP, bitter gourd peroxidase; S-BGP, soluble bitter gourd peroxidase; I-BGP, immobilized bitter gourd peroxidase; HRP, horseradish peroxidase; THF, tetrahydrofuran; DEAE, diethyl aminoethyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; CTAB, cetyl trimethyl ammonium bromide; GdnCl, guanidinium hydrochloride; ELISA, enzyme linked immunosorbent assay; TBS, Tris buffer saline; TBS-T, Tris buffer saline Tween-20

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1999; Mislovicova et al., 2000; Jan et al., 2001; Bucur et al., 2005; Andreescu and Marty, 2006).

Bioaffinity-based procedure gives oriented immobilization to enzymes that facilitates good expression of activity and possibility of direct enzyme immobilization from partially purified preparation or even crude homogenate (Akhtar et al., 2005a; Khan et al., 2005). Among the bioaffinity pairs the antigen and antibody pair is highly specific and this pair could be exploited for the immobilization of all kinds of enzymes (Saleemuddin, 1999).

Here, an effort has been made to immobilize peroxidase directly from the ammonium sulphate fractionated proteins of bitter gourd on an immunoaffinity support (IgG-Sepharose 4B). The stability of immunoaffinity immobilized bitter gourd peroxidase (BGP) has been investigated against heat, chaotropic agents (urea and guanidinium chloride), detergents (CTAB and Surf Excel), proteolytic enzyme (trypsin) and water-miscible organic solvents (propanol, THF and dioxane). Immobilized BGP preparation was also compared for its stability with its soluble form.

2. Materials and methods

2.1. Materials

Sepharose 4B, Sephacryl S-100, and cyanogen bromide (CNBr) were purchased from Sigma Chem. Co. (St. Louis, MO) USA. Con A-Sepharose was the product of Genei, India. DEAE-cellulose, GdnCl, chemicals and reagents used in electrophoresis and immunodiffusion were obtained from SRL Chemicals, Mumbai, India. All other chemicals and reagents were of analytical grade. Bitter gourd was obtained from the local market.

2.2. Immunization

Glycosylated isoform of BGP was isolated to homogeneity by using a three-step purification scheme involving ammonium sulphate fractionation, gel filtration on Sephacryl S-100 and bioaffinity column chromatography on concanavalin A (Con A)-Sepharose. Purified glycosylated BGP was injected into healthy male albino rabbits weighing 2–3 kg for the production of anti-BGP polyclonal antibodies. The animals received subcutaneously 300 μg of BGP dissolved in 0.5 mL of 20 mM sodium phosphate buffer, pH 7.2, mixed and emulsified with equal volume of Freund's complete adjuvant as first dose (Jan et al., 2001). Booster doses of 150 μg of BGP mixed and emulsified with Freund's incomplete adjuvant were administered weekly after resting the animal for 15 days. After each booster dose blood was collected from the ear vein of the animal and allowed to clot at room temperature for 3 h. Serum was collected by centrifugation at 1600 × *g* for 20 min at 4 °C and later it was decomplimented by incubating at 56 °C for 30 min. After adding sodium azide (0.2%) serum was stored at −20 °C.

2.3. Purification and characterization of polyclonal antibodies

The antiserum was fractionated with 20–40% ammonium sulphate. The sample was kept overnight with constant stirring at 4 °C to precipitate out proteins. The precipitated proteins were collected by centrifugation at 1600 × *g* for 20 min at 4 °C. The pellet obtained was re-dissolved in a minimum volume of 20 mM sodium phosphate buffer, pH 7.2 and was subjected to extensive dialysis against the same buffer to remove traces of ammonium sulphate.

Antibodies against BGP were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate precipitated antiserum was passed through DEAE-cellulose column (1.20 cm × 10.0 cm) and the fractions containing purified antiperoxidase antibodies were pooled for further use (Khan et al., 2005).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on (12.5% gel) under denaturing conditions and native PAGE on (7.5% gel) were run to separate proteins present in DEAE-cellulose purified antiperoxidase antibodies Laemmli (1970). The staining and de-staining was also performed by the same procedure. Molecular weight marker proteins (myosin, 205 kDa; β galactosidase, 116 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa; lysozyme, 14.3 kDa) were also run in one lane adjacent to the purified IgG.

2.4. Immunodiffusion

Ouchterlony double immunodiffusion was used to confirm the presence of antibodies against BGP. Immunodiffusion was performed in 1.0% (w/v) agarose prepared in normal saline (Jan et al., 2001). The cross-reactivity of antibodies was also checked against the horseradish peroxidase (HRP). The purified antiperoxidase antibodies were employed for preparing immunoaffinity support.

2.5. Direct binding ELISA

Polystyrene (96-well) microtitre plate was coated with 100 μL of antigen (BGP) at a concentration of 10 μg/mL prepared in antigen coating buffer (bicarbonate buffer, 50 mM, pH 9.6) and then incubated for 2 h at 37 °C followed by overnight storage at 4 °C. The wells were then washed three times with TBS-T buffer. The unoccupied sites were blocked with 2% fat milk in TBS (150 μL each well) followed by incubation for 5–6 h at room temperature. The wells were then washed twice with TBS-T. The test and control wells were then diluted with 100 μL of serially diluted serum. Each dilution was in TBS buffer. Serially diluted blanks corresponding to each dilution were also present. The plate was then incubated for 2 h at room temperature and overnight at 4 °C. The plate was washed again with TBS-T buffer (five times). Bound antibodies were assayed with an appropriate conjugate of anti-rabbit alkaline phosphatase (1:3000), 100 μL of it was coated in each well and kept at room temperature for 2 h. Washing of the plate with TBS-T (five times) and with distilled water (two times) was followed by addition of *p*-nitrophenyl phosphate (50 μg/100 μL) in each well and incubation at 37 °C for 30–45 min. The absorbance of each well was monitored at 405 nm on a Lab system ELISA Reader.

2.6. Preparation of immunoaffinity support for the immobilization of BGP

Sepharose 4B (5.0 g) was activated as described by Porath et al. (1967). The Sepharose 4B was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 10.0 mL of 1.0 M Na_2CO_3 and stirred slowly by placing on a magnetic stirrer at 4 °C for 30 min. Cyanogen bromide (CNBr) (1.0 g) dissolved in 1.0 mL of acetonitrile was added to the beaker containing Sepharose 4B and was again stirred for 10 min in cold. The whole mass was transferred immediately to a sintered funnel and washed thoroughly with sufficient volume of 0.1 M bicarbonate buffer, pH 8.5, distilled water, and again with same buffer. Washed activated Sepharose 4B was dried and re-suspended in 5.0 mL of 0.1 M bicarbonate buffer, pH 8.5. Purified antibodies (60 mg) were mixed with 5.0 mL of activated Sepharose 4B and stirred overnight in cold. Sepharose bound antibodies was centrifuged to remove unbound antibodies. Antibody bound matrix was extensively washed with 0.1 M bicarbonate buffer, pH 8.5 containing 1.0 M NaCl. This washed suspension was treated with 7.0 mL of 0.1 M glycine for 2 h at 4 °C. Antibody bound matrix was successively washed with 0.1 M sodium bicarbonate buffer, pH 8.5 containing 1.0 M NaCl, distilled water and finally with 50 mM sodium phosphate buffer, pH 7.0. The quantity of bound antibody was calculated by subtracting the unbound protein in the washings from that of total added protein.

2.7. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (50 g) was homogenized in a blender with 100 mL of 100 mM sodium acetate buffer, pH 5.6. The homogenate was then passed through a muslin cloth and a filtrate was obtained. The filtrate was further centrifuged at 10,000 × *g* on a Remi R-24 Cooling Centrifuge. The solution thus obtained was

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subjected to salt fractionation by adding 50–80% (w/v) $(NH_4)_2SO_4$. The mixture was stirred overnight at 4 °C to obtain the maximum precipitate. The precipitate was collected by centrifugation at 10,000 × *g* on a Remi R-24 Cooling Centrifuge. The obtained precipitate was re-dissolved in 50 mM sodium acetate buffer, pH 5.6 and it was dialyzed against the same buffer (Akhtar et al., 2005a).

2.8. Purification of BGP by gel filtration and bioaffinity chromatography

The salt fractionated and dialyzed BGP was filtered through Whatman filter paper. The enzyme was then concentrated. The Sephacryl S-100 column (49 cm × 1.7 cm) was equilibrated with 100 mM sodium acetate buffer, pH 5.6. The ammonium sulphate fractionated and dialyzed BGP was then loaded on the column. Fractions of 2.0 mL were collected using 100 mM sodium acetate buffer, pH 5.6. The flow rate of the column was 8 mL/h. The elution volume from the column was 114 mL. Protein concentration and peroxidase activity were determined in all collected fractions. Con A-Sepharose column was equilibrated with 100 mM sodium acetate buffer, pH 5.6 containing 1 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.15 M NaCl. The fractions obtained from the main peak of Sephacryl S-100 column exhibiting peroxidase activity were then pooled and passed through it. Bioaffinity adsorbed proteins were eluted by passing 100 mM sodium acetate buffer, pH 5.6 containing 0.5 M methyl α-D-mannopyranoside. The flow rate of Con A-Sepharose column was 15 mL/h. The elution volume from the column was 44 mL. Both the columns were run at a temperature of 25 °C.

2.9. Immobilization of BGP on IgG-Sepharose 4B

Ammonium sulphate fractionated and dialyzed enzyme solution (4 mL, 2925 U BGP) was mixed with 4.0 mL of CNBr activated IgG-Sepharose 4B. The mixture was stirred overnight at 4 °C. The gel was then thoroughly washed with 50 mM sodium phosphate buffer, pH 7.0 to remove unbound enzyme (Jan and Husain, 2004).

2.10. Effectiveness factor (η)

The effectiveness factor (η) value of the immobilized preparation represents the ratio of actual and theoretical activity of the immobilized enzyme (Muller and Zwing, 1982).

2.11. Measurement of peroxidase activity

Peroxidase activity was determined from the change in the optical density (λ_{460} nm) by measuring the initial rate of oxidation of 6 mM *o*-dianisidine–HCl in the presence of 18 mM hydrogen peroxide in 100 mM sodium acetate buffer, pH 5.6, for 15 min at 37 °C (Matto and Husain, 2006).

The immobilized preparation was continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparations (Akhtar et al., 2005b).

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 μmol of *o*-dianisidine–HCl per min at 37 °C into colored product ($\varepsilon_m = 30{,}000\ M^{-1}\ L^{-1}$).

2.12. Estimation of protein concentration

The protein concentration was estimated by method of Lowry et al. (1951). Bovine serum albumin was used as a standard protein.

2.13. Effect of temperature

Soluble and immobilized BGP (0.4 U/mL) were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.6. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and then the peroxidase activity was determined.

2.14. Effect of urea

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of urea (2.0–8.0 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 °C. Peroxidase activity was determined after each incubation period. The activity of urea untreated enzyme was considered as control (100%) for calculating percent activity.

2.15. Effect of GdnCl

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of GdnCl (0.0–3.5 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 °C. Peroxidase activity was determined after each incubation period. The activity of the GdnCl untreated enzyme was considered as control (100%) for calculating percent activity.

2.16. Effect of trypsin

Soluble and immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of trypsin (0.25–2.5% mg/mL) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. For calculating the percent activity trypsin untreated samples were considered as control (100%).

2.17. Effect of organic solvents

Soluble and immobilized BGP were incubated with propanol/THF/dioxane (0–60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of soluble and immobilized BGP in assay buffer without any water-miscible organic solvent was taken for the calculation of percent activity as control (100%). Peroxidase activity was assayed at all the indicated organic solvent concentrations.

2.18. Effect of detergents

Soluble and immobilized BGP (0.4 U/mL) were incubated with CTAB and Surf Excel (0.2–2.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of soluble and immobilized BGP in assay buffer without any detergents was taken for the calculation of percent activity as control (100%) separately. Peroxidase activity was determined at all the indicated detergent concentrations.

3. Results

3.1. Production and purification of anti-BGP polyclonal antibodies

BGP purified to homogeneity was highly immunogenic in rabbits. Purified antibodies raised against BGP gave a clear single precipitin line with this enzyme as evident from Fig. 1. There was a cross-reaction with HRP (data not given). The glycan structures on plant glycoproteins (including peroxidases) are highly antigenic (van Huystee and McManus, 1998). These findings suggested that the peroxidase was immunogenic in rabbits. DEAE-cellulose purified IgG loaded on SDS-PAGE clearly showed two bands, which corresponded to heavy and light chains of the antibody (Fig. 2a). The native PAGE showed a single band (Fig. 2b) and this result supported the purity of the purified antibodies. The titer obtained through direct binding ELISA was greater or equal to 51,200 (Fig. 3). Overnight incubation of fixed amount of BGP with increasing concentration of purified IgG exhibited no loss in enzyme activity. These observations further supported

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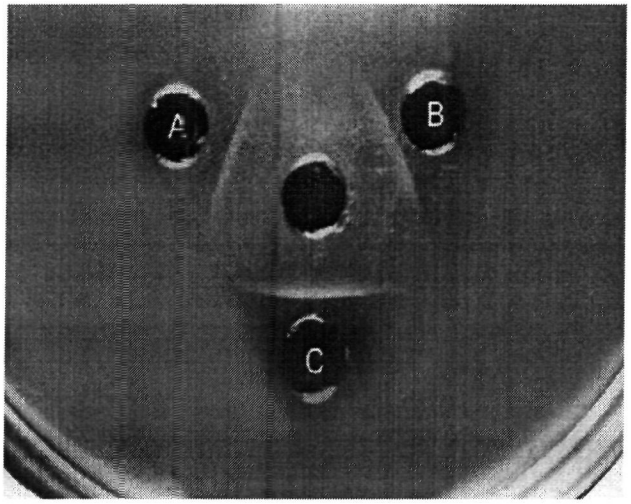

Fig. 1. Ouchterlony double immunodiffusion of the BGP against the purified anti-BGP antibody. Immunodiffusion was performed in 1% (w/v) agarose gel prepared in normal saline. The central well-contained 60 μg ammonium sulphate fractionated BGP while the outer wells contained 40 μg anti-BGP antibodies.

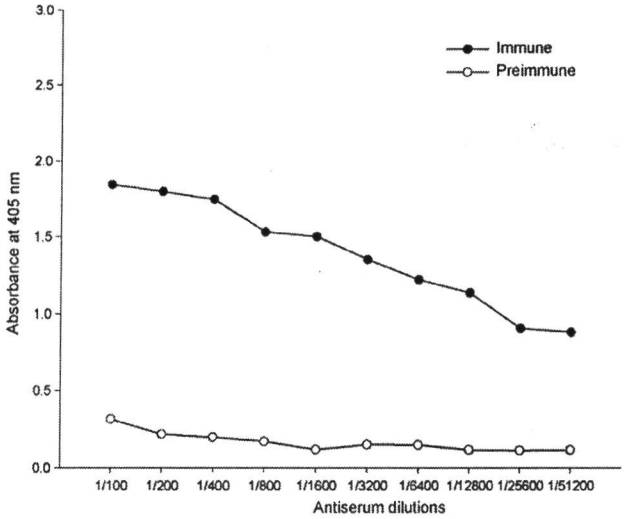

Fig. 3. Direct Binding ELISA. Serially diluted antiserum and pre immune serum were incubated in a polystyrene microtitre plate with the antigen (BGP). Serially diluted blanks corresponding to each dilution were also present. The absorbance of each well (after addition of substrate, *p*-nitrophenyl phosphate) was monitored at 405 nm on Lab systems ELISA Reader.

that the antiserum raised against the purified BGP contained only non-inhibitory antibodies (data not given).

3.2. Immobilization of BGP on the anti-BGP IgG-Sepharose 4B

The IgG isolated by ammonium sulphate fractionation and ion exchange chromatography were used for the construction of Sepharose 4B-antiBGP immunoaffinity support for the immobilization of BGP from the partially purified BGP preparation. The calculated amount of bound antibodies was 48.45 mg/5 mL of Sepharose 4B. Anti-BGP polyclonal antibody bound Sepharose 4B specifically retained peroxidase nearly 615 U/mL of the matrix. The preparation thus obtained was highly active and exhibited very high effectiveness factor η as 0.96 (Table 1).

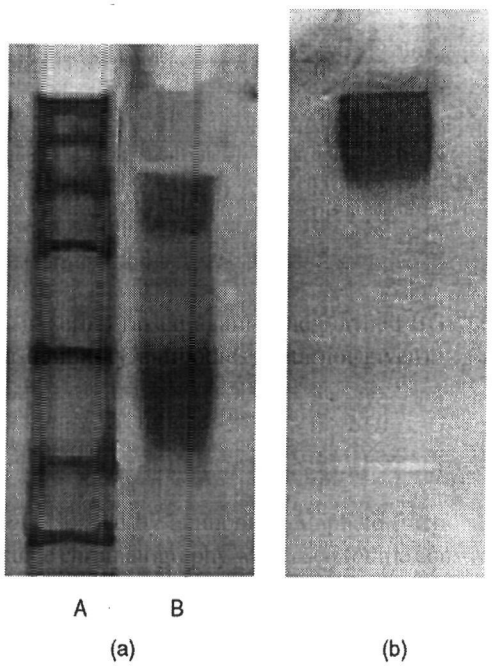

Fig. 2. (a) SDS-PAGE for the purified anti-BGP polyclonal antibodies. SDS-PAGE (12.5%, w/v) was used to characterize the purified antibodies. Lane A represents the marker proteins (30 μg was loaded). Lane B represents the DEAE fraction, 30 μg was loaded in the well. (b) Native PAGE for purified anti-BGP polyclonal antibodies. Non-denaturing, native PAGE (7.5%) was run. DEAE fraction 30 μg was loaded in the well.

3.3. Stability properties of soluble and immobilized BGP

In order to monitor the compatibility of immobilized BGP preparation in various applications, the investigations of its stability against different physical and chemical parameters is necessary.

The thermal stability of soluble and immunoaffinity bound BGP was monitored after incubating at 60 °C for various time intervals (Fig. 4). Immobilized BGP exhibited nearly 44% of the original activity even after 2 h incubation at 60 °C while the soluble enzyme lost 83% activity under similar experimental conditions. Immunoaffinity bound BGP exhibited a marginal broadening in temperature–activity profile, there being no difference in activity between 30 and 40 °C whereas free BGP had a temperature-optima at 40 °C (data not given).

The effect of different concentrations of urea on soluble and immobilized BGP is depicted in Fig. 5. Immobilized enzyme did not lose any activity till 2.0 M urea. At 8.0 M urea the soluble enzyme retained 86% of the activity whereas the immobilized enzyme preparation exhibited 93% of the initial activity. Effect of GdnCl concentrations on both soluble and immobilized BGP is shown in Fig. 6. Immobilized enzyme retained 38% activity at 3.0 M GdnCl whereas soluble enzyme exhibited only 12% of the initial enzyme activity. Immobilized preparation was more stable than its soluble form at all the concentrations indicated.

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Table 1
Immobilization of BGP on IgG-Sepharose 4B support

Amount of enzyme loaded (X) (U)	Amount of enzyme activity in washes (Y) (U)	Activity bound/mL of IgG-Sepharose 4B (U)			% Activity yield ($B/A \times 100$)
		Theoretical ($X - Y = A$) (A)	Actual (B)	Effectiveness factor (η) (B/A)	
731	90	641	615	0.96	96

Each value represents the mean for three-independent experiments performed in duplicate, with variations not exceeding 5% of the mean values. Peroxidase activity was assayed according to the procedure described in the text.

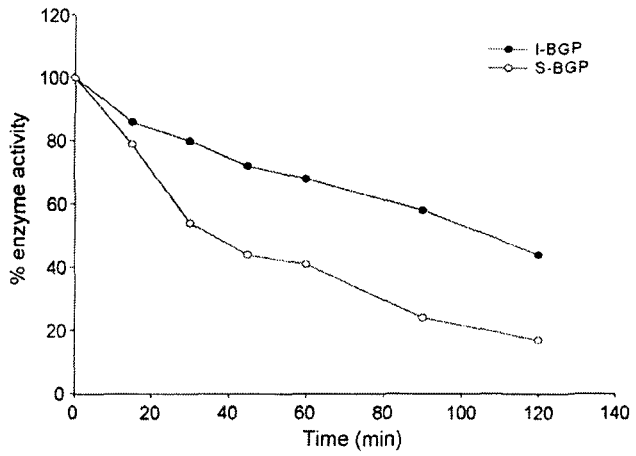

Fig. 4. Thermal denaturation plot of soluble and immobilized BGP. Soluble and immobilized BGP preparations (0.4 U/mL) were incubated at 60 °C for various time intervals in 100 mM sodium acetate buffer, pH 5.6. Aliquots of appropriate amount were removed at different time intervals and the enzyme activity was determined. For calculating the percent activity untreated samples were considered as control (100%).

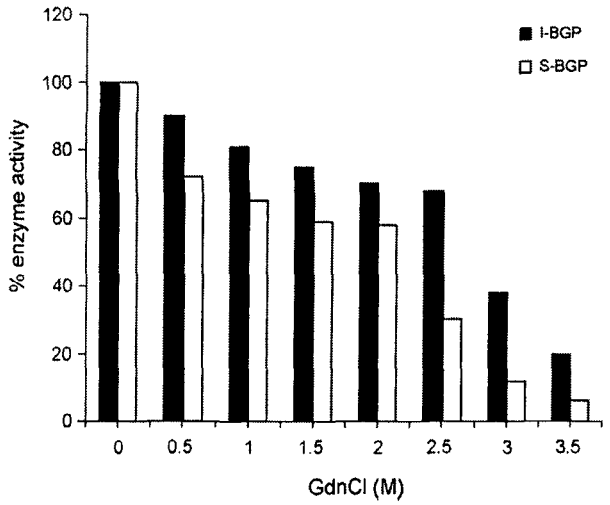

Fig. 6. Effect of GdnCl on soluble and immobilized BGP. Soluble and immobilized BGP (0.4 U/mL) were incubated in 100 mM, sodium acetate buffer, pH 5.6 containing (0–3.5 M) GdnCl at 37 °C for 1 h. Enzyme activity was determined as mentioned in the text. For calculating the percent activity untreated samples were considered as control (100%).

Soluble and immobilized BGP was treated with increasing concentration of trypsin (0–2.5%, w/v) for 1 h at 37 °C (Fig. 7). The activity of immobilized BGP enhanced to 120% after exposure to 1.0% trypsin whereas the soluble enzyme exhibited a loss of 10% of the initial activity under the similar incubation conditions. At 2.5% trypsin exposure immobilized BGP preparation retained 93% activity whereas the soluble counterpart lost 50% activity under identical treatment. The immobilized BGP showed greater stability at almost all the concentrations of trypsin.

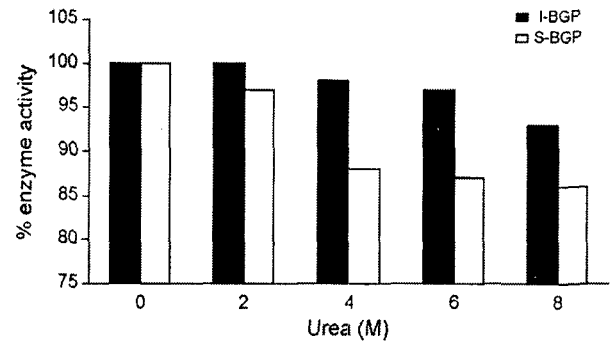

Fig. 5. Effect of urea on soluble and immobilized BGP. Soluble and immobilized BGP (0.4 U/mL) were incubated in 2.0 M, 4.0 M, 6.0 M and 8.0 M urea in 100 mM, sodium acetate buffer, pH 5.6 at 37 °C for 2 h. Enzyme activity was determined as mentioned in the text. For calculating the percent activity untreated samples were considered as control (100%).

The soluble and immunoaffinity bound BGP were treated with various concentrations of water-miscible organic solvents (0–60%, v/v) for 1 h at 37 °C (Table 2). Immobilized BGP retained 69% of the original activity when it was exposed to

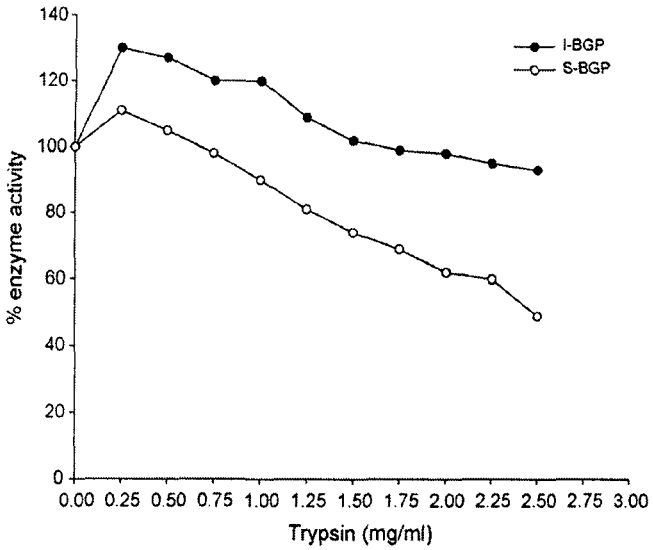

Fig. 7. Effect of trypsin on soluble and immobilized BGP. Soluble an immobilized BGP (0.4 U/mL) were incubated with increasing concentrations of trypsin (0.25–2.5% mg/mL) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. For calculating the percent activity untreated samples were considered as control (100%).

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Table 2
Effect of water-miscible organic solvents on soluble and immobilized BGP

% Organic solvent	% Residual enzyme activity					
	Propanol		THF		Dioxane	
	S-BGP	I-BGP	S-BGP	I-BGP	S-BGP	I-BGP
0	100	100	100	100	100	100
10	69	82	80	93	64	85
20	57	79	70	90	59	81
30	44	69	65	86	52	71
40	39	60	58	78	41	70
50	34	58	50	65	32	68
60	28	52	45	60	20	64

Soluble and immobilized BGP were incubated with propanol/THF/dioxane (0–60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any water-miscible organic solvent was taken as control (100%). Each value represents the mean for three-independent experiments performed in duplicates, with variations not exceeding 5% of the mean values.

30% (v/v) propanol for 1 h while its soluble counterpart lost nearly 56% of its initial activity under identical treatment. Exposure of IgG-Sepharose 4B bound BGP to higher concentrations of propanol resulted in the retention of more than 50% activity whereas the soluble counterpart showed a rapid loss in catalytic activity, retaining only 28% activity at 60% (v/v) of propanol. The immobilized BGP after treatment with 30% (v/v) THF retained 86% of the original activity; however the soluble BGP under similar exposure showed only 65% of its activity. More than 50% of the original activity was retained in case of immobilized enzyme after treatment with 60% (v/v) dioxane while the soluble enzyme lost nearly 80% of its activity at the same concentration of dioxane.

The effect of different detergents (0.2–2.0%, w/v) on activity of both preparations of BGP after 1 h of incubation at 37 °C is shown in Table 3. In the presence of CTAB both immobilized and soluble BGP showed an enhancement in their catalytic activity. However, the enhancement in the activity for the immobilized preparation was higher than the soluble form of the enzyme at each concentration. For example, IgG-Sepharose 4B bound BGP showed an enhancement of 150% and 133% at 1.0% (w/v) and 2.0% (w/v) of CTAB, respectively, whereas the soluble counterpart showed activation up to 119% and 104% of the initial activity of BGP, respectively, under identical conditions. The immobilized enzyme retained 91% of its original activity at 1.0% (w/v) of Surf Excel exposure whereas the soluble counterpart exhibited only 62% activity under similar treatment. At 2.0% (w/v) Surf Excel, immobilized enzyme retained 48% activity, whereas the soluble enzyme exhibited a loss of nearly 88% of its initial activity.

Table 3
Effect of detergents on soluble and immobilized BGP

Detergent (%, v/v)	Percent remaining activity			
	CTAB		Surf Excel	
	S-BGP	I-BGP	S-BGP	I-BGP
0.2	149	178	103	111
0.4	142	178	90	102
0.6	130	159	82	98
0.8	125	157	70	95
1.0	119	150	62	91
1.2	116	148	50	77
1.4	112	140	48	70
1.6	109	137	36	58
1.8	105	133	27	50
2.0	104	133	12	48

Soluble and immobilized BGP (0.4 U/mL) were incubated with CTAB and Surf Excel (0.2–2.0%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined in all the tubes as described in the text. Each value represents the mean for three-independent experiments performed in duplicate with the average deviation not exceeding more than 5%.

3.4. Kinetic analysis

K_m obtained from Lineweaver Burk plots was 1.3 mM for both soluble and immobilized preparations. The k_{cat} for soluble BGP was 2.7 s^{-1} while that for immobilized preparation was 2.0 s^{-1}.

4. Discussion

The enzyme electrodes based on immobilized peroxidase have been successfully employed for measuring various types of chemical pollutants (Alpeeva et al., 2005; Gaspar et al., 2000; Wang et al., 2001; Schumacher et al., 2001). The rare availability and high cost of the commercially purified HRP has limited its use for various reasons. In order to circumvent such problem, it is advantageous to use ammonium sulphate fractionated bitter gourd proteins instead of pure enzyme for the immobilization of peroxidase. Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure (Muller and Zwing, 1982). A large number of earlier studies have described that the enzymes immobilized on the antibody support showed very high effectiveness factor (Jan et al., 2001; Jan and Husain, 2004). The specific binding of peroxidase to the immunoaffinity support directly from the ammonium sulphate precipitated proteins of bitter gourd is significantly useful in reducing the cost of immobilized enzyme preparation.

Immunoaffinity bound BGP was significantly more stable against urea and GdnCl denaturation and it suggested that such preparation could be exploited even in the presence of such type of chaotropic agents. Urea and GdnCl are very frequently used as strong denaturants of proteins, which have been shown to undergo pronounced structural changes at concentrations greater than 1.0 M of these denaturants (Tanford, 1970). However, the action mechanism of urea induced denaturation of proteins structure has not been clearly known, several earlier studies have proposed that protein is unfolded by the direct interaction of urea molecule with a peptide backbone via hydrogen bonding/hydrophobic interaction, which contributes to the maintenance of protein conformation (Makhatadze and Privalor, 1992).

Immobilized BGP exhibited remarkably high stability against the water-miscible organic solvents induced inactivation. The

significance of support matrices for the utilization of enzymes in organic solvents has already been recognized (Batra and Gupta, 1994; Kulshrestha and Husain, 2006) and proteinic supports may be particularly useful (Jan et al., 2001; Akhtar et al., 2005a; Khan et al., 2005; Matto and Husain, 2006). More recently our group has shown that IgG-Co^{2+}-Sepharose/IgG-Sepharose bound glucose oxidase was significantly stable against the inactivation mediated by pH, urea, heat and water-miscible organic solvents (Jan et al., 2001; Jan and Husain, 2004). The significance of other supports in the stabilization of peroxidases against water-miscible organic solvents has also been described (Kulshrestha and Husain, 2006).

Immunoaffinity bound BGP showed an enhancement in its activity even when it was exposed to a very high concentration of cationic detergent, CTAB while this activation was decreased in case of soluble BGP (Table 3). We have earlier shown that BGP immobilized on Con-A-Sephadex support was also activated by the exposure of lower concentration of detergent (Akhtar et al., 2005a). It has been shown by other workers that some enzymes can show enhanced activity in water/detergent media owing to the positive interactions between enzyme and detergent (Viparelli and Francesco, 1999). This bioaffinity bound BGP preparation was quite resistant to the inactivation induced by anionic detergent like SDS (Akhtar et al., 2005a). In view of its high stability in the presence of detergents, it is possible to use such preparations for the treatment of wastewater contaminated with various organic pollutants along with detergents. Some earlier reports indicated that the peroxidases immobilized by other methods also resulted into stabilization against the exposure caused by detergents (Kulshrestha and Husain, 2006). The K_m values determined for immobilized and soluble BGP were same while the k_{cat} for soluble BGP was slightly higher than immobilized preparation.

Storage stability studies performed in our lab indicated that Immobilized BGP was quite stable and retained 93% of activity even after 2 months of storage at 4 °C.

This study suggested that the enzyme bound to the IgG-Sepharose 4B support could be used more effectively for the conversion of various compounds, which are insoluble or sparingly soluble in aqueous environment in the presence of high concentrations of water-miscible organic solvents and detergents. The generally observed higher stability of the immunoaffinity bound BGP against various forms of inactivation may be related to the specific and strong binding of enzyme with antibody support which prevents the unfolding/denaturation of enzyme. In view of the improved stability of immunoaffinity bound BGP against heat, pH, chaotropic agents, detergents, proteolytic enzyme and water-miscible organic solvents, it may find a large number of applications especially in the construction of enzyme-based analytical devices for clinical, environmental and food technology (Gaspar et al., 2000; Alpeeva et al., 2005).

The main objective of this work was to cut down the cost of the enzyme purification and to immobilize the enzyme at the step of purification, which can turn out to be of great interest in the area of clinical and environmental analysis. This immobilized preparation was remarkably stable against the inactivation mediated by heat, pH, chaotropic agents, detergents and water-miscible organic solvents. Such preparations could be easily employed in the analysis of water soluble and insoluble compounds, synthesis of novel organic compounds, solvent engineering and protein engineering.

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